610.5
J65b
Medical Lib.
FEB 1 1933

VOLUME LII NUMBER 2

BULLETIN of the JOHNS HOPKINS HOSPITAL

THE PUBLICATION OF THE MEDICAL SCHOOL AND HOSPITAL
SUPPORTED BY THE DE LAMAR FUND OF THE JOHNS HOPKINS UNIVERSITY

February, 1933

Skin-reacting Substances Present in the Urine during Acute Streptococcal Infections. E. T. Conybeare, M.R.C.P. 119
Observations upon a Case of Post-operative Hypoparathyroidism. Read Ellsworth 131
Studies in Experimental Syphilis. X. Observations on Cross-inoculations with Heterologous Strains of Syphilitic Virus. Alan M. Chesney, M.D., Thomas B. Turner, M.D., and Franklin H. Grauer, M.D. 145
Hyperproteinemia Associated with Multiple Myeloma, with Report of a Case in Which an Extraordinary Hyperproteinemia Was Associated with Thrombosis of the Retinal Veins and Symptoms Suggesting Raynaud's Disease. Maxwell M. Wintrobe, M.D., Ph.D., and Mary V. Buell, Ph.D. 156
Studies on an Urea-splitting Enzyme Found in the Gastric Juice. Lay Martin 166

Supplement—BULLETIN OF THE INSTITUTE OF THE HISTORY OF MEDICINE

The Johns Hopkins University

VOLUME I, NUMBER 2

Activities of the Institute of the History of Medicine 49
The Warrington Academy (1757–1786) and Its Influence upon Medicine and Science. John F. Fulton, M.D. 50
A New and Hitherto Unpublished Portrait of Marcello Malpighi. Michel Pijoan 81

PUBLISHED MONTHLY

THE JOHNS HOPKINS PRESS, Baltimore, Maryland

Made in United States of America

BULLETIN

OF

THE JOHNS HOPKINS HOSPITAL

MANAGING EDITOR, E. Cowles Andrus

EDITORIAL BOARD

Charles R. Austrian
Thomas R. Boggs
Edward P. Carter
Arnold R. Rich
Paul G. Shipley
Frank R. Smith

All correspondence relating to the publication of papers should be addressed to The Editor, Bulletin of The Johns Hopkins Hospital, Baltimore, Md.

Books for reviews should be sent to The Editor at the above address.

Twenty-five reprints, with covers, of articles will be furnished to contributors free of cost. An order slip for additional reprints, with a table showing cost, is sent with proof.

Correspondence concerning business matters should be sent to The Johns Hopkins Press, Baltimore, Maryland, U. S. A.

This periodical is issued monthly. Two volumes a year are issued, each consisting of approximately 400 pages, and beginning in January and July, respectively.

Subscription price. In the United States and its possessions $6.00 per year. In Canada $6.25 per year; in foreign countries $6.50 per year. Prices for back volumes and back single numbers on application. Single numbers of the current volume, when available, $.75. Prices are net, postpaid.

New Subscriptions and renewals are entered to begin with the first issue of the current volume. Should any issue of the current volume be out of print at the time the subscription order is received, the pro rata value of such numbers will be credited to the next volume, and the renewal invoice therefore adjusted accordingly.

Subscriptions should be renewed promptly. To avoid a break in your series, subscriptions should be renewed promptly. The publisher cannot guarantee to supply back issues on belated renewals.

Subscriptions, new or renewal, may be sent to the Publishers or ordered through your agent.

Agents for the British Empire, except Canada: Baillière, Tindall & Cox, 8 Henrietta St., Covent Garden, W.C. 2, London.

THE JOHNS HOPKINS PRESS

BALTIMORE, MARYLAND, U. S. A.

Entered as second-class matter at the Baltimore, Md., Postoffice. Acceptance for mailing at special rate of postage provided for in Section 1103, act of October 3, 1917. Authorized on July 3, 1918

WILLIAM SYDNEY THAYER

Due to an error the portrait of Dr. Thayer in the January number of the Bulletin of the Johns Hopkins Hospital was reversed in reproduction. The above is offered in correction of this error.

SKIN-REACTING SUBSTANCES PRESENT IN THE URINE DURING ACUTE STREPTOCOCCAL INFECTIONS[1]

E. T. CONYBEARE, M.R.C.P.[2]

From the Biological Division of the Medical Clinic, the Johns Hopkins Hospital and University

In 1924 Trask and Blake (1) considered they had demonstrated the presence of a streptococcal toxin in the urine of patients with scarlet fever. By the injection of small quantities of such urine intradermally in Dick-positive subjects they obtained a marked skin reaction which was apparently due to a substance neutralisable by anti-scarlatinal serum.

In numerous publications Longcope (2) has brought forward convincing evidence that a high proportion of patients in the early stages of haemorrhagic Bright's disease show the Dick-positive skin condition in a very striking form and react strongly to high dilutions of the filtrate from cultures of many β-hemolytic strains of streptococci.

At the outset of the present investigation an attempt was made to confirm the work of Trask and Blake, the test subjects used being those patients with acute haemorrhagic nephritis who were found to possess a high degree of skin sensitisation to streptococcal culture filtrates during the course of their special examination at the Osler Clinic of the Johns Hopkins Hospital. In view of the difficulty from pseudo-reactions experienced with direct urinary injections, a modified procedure was adopted in which the filtered urine was first extracted in a manner recently described by Barber and Oriel (3). This method provides a means of obtaining colloidal matter from biological fluids without the use of large quantities of alcohol and was found to be effective in removing the toxic factor from culture filtrates of β-hemolytic streptococci.

The investigation was later extended to include those patients with acute pharyngitis and other infectious conditions associated with hemolytic streptococci of the beta type who were admitted to the

[1] Submitted for publication July 29, 1932.

[2] John D. Archbold Fellow in Medicine.

isolation ward of the Johns Hopkins Hospital during the first four months of the present year.

PREPARATION OF THE URINARY EXTRACT

A fresh urine specimen centrifuged free of gross suspended matter was passed through a Berkefeld N candle filter. Not less than 150 cc. of the filtrate acidified to pH 3 by the addition of 25 per cent sulphuric acid was placed in a separating funnel and cooled to about 15°C. The addition of 30 to 40 cc. of purified ether, following by shaking, produced an emulsion which separated into two or more layers at a rate apparently dependent on the amount of colloidal material present. With a highly albuminous urine layer formation frequently took nearly an hour, whereas in the case of a dilute non-albuminous specimen layering was usually complete in a few minutes. As soon as practicable the lower urinary layer was withdrawn and set aside for a second extraction. The upper ethereal layers were no longer clear but opalescent and often somewhat viscous, a result apparently of the concentration of a large proportion of the colloidal material in the extensive increase of urine-ether interface produced by the initial shaking. This material was then precipitated from the upper layers by the addition of an equal volume of absolute alcohol. Separation of the precipitate from the alcohol-ether mixture was accomplished by centrifugation and evaporation at room temperature. As much of the precipitate as would dissolve or go into stable suspension was then taken up in 25 cc. of the phosphate buffer solution (pH 7.4) described by Evans (4). A micro-Kjeldahl estimation of the nitrogen content of this buffer solution extract was then made on a 5 cc. sample. The results of this estimation, which was made in the laboratories of the Chemical Division of the Osler Medical Clinic, varied with different urines over a range of from 10 to 150 mgm. N per 100 cc. In all cases a final dilution of the remaining solution with the phosphate buffer to a nitrogen content of 5 mgm. per 100 cc. was made for use in skin testing, experience with filtrates from cultures of β-hemolytic streptococci having shown that a dilution of this strength as regards nitrogen produced sufficiently large reactions when injected intradermally into sensitive nephritic patients.

SCARLET FEVER PATIENTS

Extracts were made in the above manner from the urine of five patients admitted to the Isolation Ward of the hospital with the clinical diagnosis of scarlet fever. The 5 mgm. dilution of each extract was injected in 0.1 cc. amounts into the skin of at least two nephritic patients known to be highly sensitive to culture filtrates of β-hemolytic streptococci, and the injection sites were observed during the following 24 hours for the occurrence of erythematous reactions of the Dick type. Injections of similar extracts prepared from the urine of clinically normal individuals were made at the same time and also observed. An area of erythema exceeding 1 cm. in diameter and appearing within 18 hours was termed a positive reaction. Such reactions were produced by the urinary extracts obtained from three of the five scarlet fever patients investigated.

Case No. 42100. *Sarsfield S.*, white male, aged 13 yrs. Clinical Diagnosis: Scarlet Fever with toxaemia of moderate severity. Extracts obtained from urine on 3rd and 4th days of illness gave positive reactions. No active extract could be prepared from the urine on the 6th and 8th days. Course subsequently complicated by unilateral acute otitis media.

Case No. 42214. *Anna K.*, white female, aged 6 yrs. Clinical Diagnosis: Scarlet Fever of moderate severity. Urinary extract made on the 3rd day of illness gave positive reactions. Recovery complicated by cervical adenitis and pyrexia continued over four weeks.

Case No. 42238. *Eugene E.*, white male, aged 5 yrs. Clinical Diagnosis: Scarlet Fever. Urine on 3rd day of illness gave an extract producing positive reactions. A unilateral otitis media complicated recovery.

No positive reactions were obtained in the remaining two cases in which the acute illness appeared to be of a milder nature and the recovery uncomplicated.

Case No. 41732. *Dorothy S.*, white female, aged 9 yrs. Clinical Diagnosis: Scarlet Fever of a mild type. Extract from urine obtained on 2nd day of illness produced no positive reactions. Recovery uncomplicated.

Case No. 39276. *William H.*, aged 23 yrs., medical student. Clinical Diagnosis: Mild Scarlet Fever. Urine extract made on the 5th day of illness completely negative. Recovery uncomplicated.

In all five of the above cases the throat cultures were repeatedly positive for β-hemolytic streptococci, while in no instance did the urine specimen from which an extract was made give a positive acetic acid and boiling test for albumin.

Intradermal injections were also made on the same test subjects with twelve extracts from urine specimens provided by ten clinically normal individuals whose throat cultures, at the time, were negative for β-hemolytic streptococci. With four of these extracts a small area of erythema, less than that regarded as a positive reaction, consistently appeared at the site of injection within six or seven hours. This faded sooner than the larger area usually produced by the extracts from the urine of the scarlet fever patients and did not on inspection at twenty-four hours show the darker central and lighter surrounding zones that were characteristic of the much more slowly disappearing reaction.

NEUTRALISATION EXPERIMENTS

The sera of convalescent scarlet fever patients were obtained and incubated for one hour at 56°C. with equal amounts of a 1:200 dilution of a standard Dick toxin prepared from the N. Y. 5 strain of streptococcus scarlatinae. The antitoxic activity of these sera was then investigated on Dick-positive subjects by the intradermal injection of the incubated mixtures.

Similar experiments were performed with the same sera and a 1:100 dilution of the toxic filtrate from the 24 hours broth culture of a β-hemolytic streptococcus isolated from the throat of a patient (Wm. McC.) with subacute glomerular nephritis.

It was found that certain sera which partly or completely neutralised the Dick toxin also contained neutralising factors for the toxic filtrate of the streptococcus isolated from the nephritic patient, while with other sera no evidence of neutralising factors for the skin toxic products of β-hemolytic streptococci could be obtained. Such sera were regarded as anti-streptococcal and non-anti-streptococcal, respectively.

A serum specimen of each of the above types was next used in neutralisation experiments with the active urinary extracts from the scarlet fever patients, and also for similar experiments with the four slightly active extracts from normal individuals.

Neutralisation experiments

SCARLET FEVER CASES	DAY OF ILLNESS	SKIN REACTION* ON TEST SUBJECTS AFTER INCUBATION OF URINARY EXTRACT WITH	
		Antistreptococcal Sera	Non-antistreptococcal Sera
No. 42100. Sarsfield S.	3rd	−	+
No. 42214. Anna K.	3rd	−	+
No. 42238. Eugene E.	3rd	−	+
Four normal individuals whose urinary extracts gave slight delayed reactions		−	−

* An area of erythema exceeding 1 cm. in diameter and appearing within 24 hours was termed a positive reaction and recorded as + in this and succeeding tables. The sign − indicates no reaction within 24 hours.

It will be seen that the active extracts from the urine of the scarlet fever patients appeared to contain a factor which was neutralised only by sera possessing anti-streptococcal properties.

PATIENTS WITH ACUTE PHARYNGITIS

Extracts were also made in a similar manner from the urine of those patients admitted to the Isolation Ward of the hospital on account of a feverish illness accompanied by sore throat. These extracts were tested by the procedure already described on the same group of skin-sensitive nephritic subjects.

In five of seven patients with acute pharyngitis, throat cultures showed that hemolytic streptococci of the β type were the predominant organisms. Positive reactions were produced by the extracts derived from four of these five patients.

Case No. 16627. *Mary C.*, colored female, aged 30 yrs. Clinical Diagnosis: Acute follicular tonsillitis. Extract from urine obtained on 2nd day of illness gave positive reactions. Subsequent course was complicated by acute haemorrhagic nephritis, albumin and red blood cells appearing in the urine on the 8th day.

Case No. 41617. *Catherine F.*, colored female, aged 17 yrs. Clinical Diagnosis: Acute follicular tonsillitis. Extract from urine obtained on the 3rd day of illness gave positive reactions. Pyrexia persisted for two weeks and recovery was complicated by otitis media.

Case No. 42501. *Grenshaw B.*, white male, aged 28 yrs. Clinical Diagnosis: Subacute pharyngitis. Extract from urine obtained on the 12th day of illness when patient was still pyrexial gave positive reactions. Course subsequently complicated by an acute non-suppurative arthritis. Later tonsils on removal grew hemolytic streptococci in pure culture.

Case No. 43261. *Ruth F.*, white female, aged 22 yrs. Clinical Diagnosis: Acute tonsillitis and pharyngitis. Extract from urine obtained on 1st day of illness when patient's pyrexial reaction was at its height gave positive reactions. Recovery was rapid and uncomplicated.

In the fifth patient, although the pyrexial reaction at the time of the throat culture was severe, the subsequent illness was of a transient nature.

Case No. 41693. *Dorcas H.*, white female, aged 25 yrs. Clinical Diagnosis: Acute tonsillitis. Extract from urine obtained on 2nd day of illness gave no positive reactions. Patient was apyrexial on the 3rd day and made an uninterrupted recovery.

In the remaining two patients the hemolytic staphylococcus appeared to be the predominant organism in the throat culture. No positive reactions were obtained with the extracts derived from the urine of these two patients.

Case No. 41998. *Verna C.*, white female, aged 24 yrs. Clinical Diagnosis: Acute tonsillitis and peritonsillar abscess. Throat cultures showed hemolytic staphylococcus and micrococcus catarrhalis only. Extract made from specimen obtained on 4th day of illness with patient pyrexial, and prior to opening of the abscess, gave no positive reactions.

Case No. 23490. *Eliz. G.*, white female, aged 21 yrs. Clinical Diagnosis: Acute tonsillitis. Extract from urine on 2nd day of illness gave no positive reactions. Hemolytic staphylococcus only in throat culture.

Albumin could not be detected by the acetic acid and boiling test in the urine specimens from which the extracts were prepared in any of the above cases.

Neutralisation experiments using anti-streptococcal and non-anti-streptococcal sera were also carried out with the active urinary extracts from the four patients with acute pharyngitis.

Neutralisation experiments

ACUTE PHARYNGITIS CASES	DAY OF ILLNESS	SKIN REACTION ON TEST SUBJECTS AFTER INCUBATION OF URINARY EXTRACT WITH	
		Antistrepto-coccal Sera	Non-antistrep-tococcal Sera
No. 16627. Mary C.	2nd	−	−
No. 41617. Catherine F.	3rd	−	−
No. 42501. Grenshaw B.	12th	−	−
No. 43261. Ruth F.	1st	+	−

It therefore appeared that the skin toxic factor contained in the urinary extracts from these cases was neutralised both by anti-streptococcal sera and by sera in which no specific antibodies for the toxins of β-hemolytic streptococci could be demonstrated by direct experiment.

ERYSIPELAS PATIENTS, AND PATIENTS WITH ALBUMINURIA

A similar procedure was carried out on the urine of three patients admitted to the Isolation Ward with a clinical diagnosis of erysipelas. The extracts obtained in all three cases gave positive reactions.

Case No. 41646. *Walter S.*, white male, aged 40 yrs. Clinical Diagnosis: Erysipelas of face. Extract from urine obtained on the 13th day of illness while patient was still pyrexial gave positive reactions. Throat culture at this time repeatedly positive for β-hemolytic streptococcus.

Case No. 41733. *Dorothy W.*, white female, aged 23 yrs. Clinical Diagnosis: Erysipelas of face. Extract from urine obtained on the 5th day of illness gave positive reactions. Throat culture positive for β-hemolytic streptococcus.

Case No. 42785. *Hattie B.*, colored female, aged 46 yrs. Clinical Diagnosis: Erysipelas of buttock. Extract from urine obtained on 2nd day of illness gave marked positive reactions. There was a chronic urinary infection with coliform bacilli present in this case. Throat culture showed β-hemolytic streptococcus.

Since the urine specimens from which certain of the above extracts were made contained large amounts of albumin, further observations were carried out with extracts prepared from the highly albuminous urine of a patient with subacute nephritis and also with extracts derived from the urine of two cases of pneumonia showing albuminuria.

Case No. 41578. *Peter A.*, white male, aged 26 yrs. Clinical Diagnosis: Subacute glomerulo-nephritis. This patient, with much oedema, great albuminuria, and relatively little elevation of the blood pressure, was considered to be in the nephrotic stage of the disease. The urine extract made from a specimen obtained three weeks following tonsillectomy when throat cultures were free of β-hemolytic streptococci gave no positive reactions on four test subjects.

Case No. 42299. *Jack J.*, colored male, aged 30 yrs. Clinical Diagnosis: Lobar pneumonia. Urine obtained on the 3rd day of illness, when the blood culture was positive for group IV pneumococcus, contained much albumin. The extract from this specimen gave no positive reactions on four test subjects.

Case No. 29790. *Mildred A.*, white female, aged 36 yrs. Clinical Diagnosis: Acute pharyngitis and otitis media, broncho-pneumonia. Throat and ear cultures were positive for β-hemolytic streptococci. The blood culture was, however, negative. Urine obtained on the 4th day contained much albumin and the extract prepared from this specimen gave positive reactions on three test subjects.

It will be seen that, of the extracts made from six albuminous urines, the four from cases associated with streptococcal infection gave positive reactions, while the remaining two did not. Further neutralisation experiments of the type described in connection with the scarlet fever and acute pharyngitis patients were also carried out with the four active extracts.

Neutralisation experiments

ERYSIPELAS CASES	DAY OF ILLNESS	SKIN REACTION ON TEST SUBJECTS AFTER INCUBATION OF URINARY EXTRACT WITH	
		Antistrepto-coccal Sera	Non-antistrep-tococcal Sera
No. 41646. Walter S.	13th	−	−
No. 41733. Dorothy W.	5th	−	−
No. 42785. Hattie B.*	2nd	+	+
Broncho-pneumonia Case No. 29790. Mildred A.	4th	−	−

* The urinary extract from the case No. 42785 (Hattie B.) was found to produce skin reactions in Dick-negative individuals and in subjects whose skins were quite insensitive to streptococcal products, a property not characteristic of the other urinary extracts giving positive skin reactions. In view of the fact that the urine from which this particular extract was prepared had been heavily infected with B. coli, it was considered that the universally toxic character of this extract was probably connected with bacterial activity in an albuminous medium.

These experiments appeared to show that anti-streptococcal sera were not specific in their neutralisation of the skin toxic factor present in the urinary extracts.

DISCUSSION

The observations recorded above show that from the urine of patients with acute streptococcal infection there may be obtained an extract which produces, when injected intradermally into susceptible subjects, an erythematous reaction similar in time of onset and appearance to that caused by the injection of diluted filtrates from culture of many hemolytic streptococci of the β type. An extract of this character would appear to be more readily obtainable when the infection is severe, as judged by clinical sequelae, and also to be connected with the earlier stages of marked pyrexial reactions.

The reaction-producing factor present in culture filtrates of β-hemolytic streptococci possesses considerable heat stability and excellent keeping properties. The activity of the urinary extracts prepared in the manner described above is not diminished, as judged from the skin reactions, by exposure to a temperature of 100°C. for five minutes, and only partially reduced by fifteen minutes at that temperature. A period of three months in cold storage produced no demonstrable impairment of activity.

The neutralisation experiments, carried out with the sera of patients convalescent from scarlatina, appear to demonstrate that the urinary extracts from the scarlet fever cases contain a skin toxic factor only neutralisable by anti-streptococcal sera. The urinary extracts from other cases of infection with β-hemolytic streptococci could not be shown in this way to contain a neutralisable factor of this kind, but appeared to possess activity of a type occasionally present to a slight degree in extracts prepared from the urine of normal individuals.

The nature of the skin reaction produced by what Okell (5) has termed the "erythrogenic toxin" of the β-hemolytic streptococcus is still in doubt. Analogy to the Schick test suggests that there exists a lack of some specific antitoxin in the blood or tissues of the susceptible individual, but the reaction obtained with streptococcal material may equally well be regarded as evidence of a hypersensitive state of the skin to bacterial protein, that is, as an allergic manifestation, and not as a toxin-antitoxin reaction.

The rash of scarlet fever is readily blanched by suitable antisera (Schultz-Charlton phenomenon) such as those obtainable from patients convalescent from scarlet fever. These sera, however, are variable in their neutralising effect on the skin reaction-producing activity of diluted culture filtrates from various strains of β-hemolytic streptococci. In some cases there is what appears to be a complete neutralisation of the reaction-producing factor but more frequently only a partial effect. Of a series of eleven culture filtrates prepared with β-hemolytic streptococci isolated from many different sources, only four were completely neutralisable as regards the property of producing a skin reaction by incubation with convalescent scarlet fever sera known to give positive blanching tests, but as it was not possible to control the quantitative factors involved it is doubtful whether much significance may be attached to this finding.

Ando (6) has recently suggested the presence of at least two skin active factors in so-called Dick toxin. One factor he considers to be partially heat labile and readily neutralisable by anti-sera, *i.e.*, to behave like the diphtheria toxin. The other factor he regards as a derivative of bacterial protein extremely heat-stable and not neutralisable by anti-toxic sera but producing skin reactions on an allergic basis in patients who have become sensitised to bacterial protein. His conclusions apply equally well to the diluted filtrates from cultures of all strains of β-hemolytic streptococci.

Derick and Fulton (7) in their recent work with a streptococcal nucleo-protein fraction of the type first described by Lancefield (8), have demonstrated in a large number of patients and normal individuals a distribution of skin sensitivity to this product which follows closely that found in the earlier observations with culture filtrates. Thus the percentage of patients with acute haemorrhagic nephritis who are sensitive to the culture filtrates is practically the same as that obtained with streptococcal nucleo-protein. The percentages of reactions in clinically normal individuals are also quite comparable. It would appear then, on the basis of these findings, that bacterial protein cannot be excluded as the active agent in most of the reactions obtained with the culture filtrates but that in some cases a readily neutralisable toxic substance may also be present.

The identity of a toxic factor present in the active extracts prepared

from the urine of scarlet fever patients with the readily neutralisable component of the Dick toxin appears to be supported by the evidence available from the above experiments. The active factor present in the urinary extracts from other cases cannot, however, on the present evidence, be connected with either the toxin or disintegration products of the β-hemolytic streptococcus, but appears to represent the excretion in much increased amounts of a toxic substance occasionally present in the urine of normal individuals. A substance of this kind has recently been described by Murray Lyon (9) and by Freeman (10) in connection with the skin reactions produced by colloidal material obtained from the urine of patients with allergic diseases such as asthma and hay fever.

SUMMARY

1. Colloidal material extracted from the urine of patients suffering from acute infectious conditions associated with β-hemolytic streptococci is capable, in a high proportion of cases, of producing a delayed erythematous reaction when injected intradermally into susceptible subjects.

2. Similar material extracted from the urine of clinically normal individuals is, by comparison on the same test subjects, relatively inactive as regards the property of producing a skin reaction.

3. Such material from the urine of scarlet fever cases appears to contain the "erythrogenic toxin" (Okell) of the β-hemolytic streptococcus.

4. The reaction-producing factor present in the material extracted from the urine of patients with acute pharyngitis and erysipelas is not shown by the experiments described to be derived from the β-hemolytic streptococcus. The possible nature of this factor is discussed.

The author desires to express his indebtedness to Dr. Warfield T. Longcope and Dr. W. S. Tillett for much kindly help and criticism.

REFERENCES

(1) Trask, J. D., and F. G. Blake: *Jour. Exp. Med.*, 1924, xl, 381.

(2) Hansen-Pruss, O. C., Longcope, W. T., and D. P. O'Brien: *Jour. Clin. Invest.*, 1929, vii, No. 4, 543.

(3) Oriel, G. H., and H. W. Barber: *Lancet*, 1930, ii, 231.
(4) Evans, A.: *Jour. Infect. Dis.*, 1922, xxx, 95.
(5) Okell, C. C.: *Lancet*, 1932, i, 815.
(6) Ando, K.: *Jour. of Immunol.*, 1930, xix, 99.
(7) Derick, C. L., and M. N. Fulton: *Jour. Clin. Invest.*, 1931, x, 121.
(8) Lancefield, R.: *Jour. Exp. Med.*, 1928, xlvii, 481.
(9) Murray Lyon, P.: *Brit. Med. Jour.*, 1932, i, 136.
(10) Freeman, J.: *Lancet*, 1932, i, 561.

OBSERVATIONS UPON A CASE OF POST-OPERATIVE HYPOPARATHYROIDISM[1]

READ ELLSWORTH

From the Medical Clinic, the School of Medicine, Johns Hopkins University and Hospital

In recent years many studies have been made of the total calcium and phosphorus metabolism in cases of parathyroid deficiency. The publication of Albright, Bauer, Ropes and Aub (1) is especially to be noted. Their observations were extended by Albright and Ellsworth (2) and by others (3).

In the present work, total metabolism studies were not attempted. The serum calcium and inorganic phosphorus were followed with care, and the effects of various therapeutic agents upon them and upon the clinical symptoms of hypoparathyroidism were noted.

CLINICAL HISTORY

The patient, H. C., History No. Unit 38380, was a colored woman 36 years of age. Admitted Oct. 13, 1931, Discharged Dec. 19, 1931. Readmitted Feb. 1, 1932, Discharged Mar. 3, 1932. About 2½ years before admission to the hospital, nervousness, palpitation, dyspnea and sweating had appeared. Three months before her admission to the medical service, she was found to have Graves' Disease, and a double partial lobectomy was performed. Following operation she was well for two weeks and then began to have epigastric distress followed by stiffness and cramps in the hands and feet. She described the attacks accurately. They were accompanied by a feeling of tenseness and general "nervousness, which later became indistinct. Four weeks after onset of these symptoms she was admitted to the metabolism ward of the Osler Clinic for study.

Upon examination the positive findings were: Scar of thyroid operation, slight cardiac enlargement, mild hypertension, blood pressure 160/110; accentuated aortic second sound; procidentia. The hands were held with the fingers extended in length, but flexed at the metacarpal joints, the thumb extended and adducted. Application of tourniquet for a few seconds gave a strong and painful Trousseau's sign (*i.e.*, exaggeration of the position described above). Chvostek's sign was positive; Pool's sign positive. Examination of the eyes revealed no cataracts.

The *diagnosis* in addition to hypertension, arteriosclerosis (mild), procidentia, was post-operative hypoparathyroidism with active tetany.

The course in the hospital is represented in chart I, and table 1.

---

[1] Submitted for publication August 12, 1932.

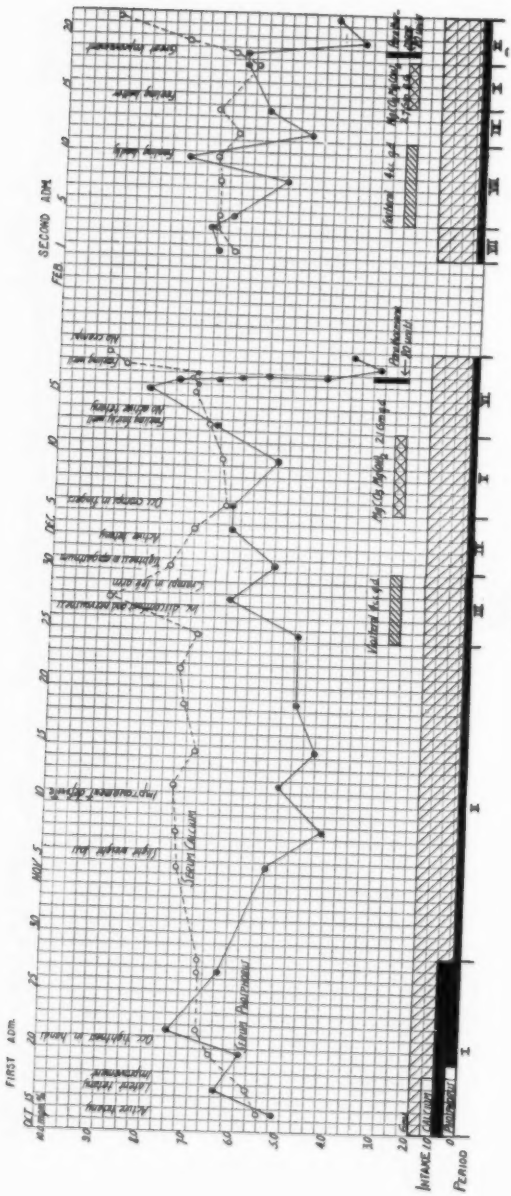

CHART I

TABLE 1

H. C. First admission

PERIOD	DAY AFTER ADMISSION	DAILY INTAKE		THERAPY	SERUM		TROUSSEAU	OTHER LABORATORY REPORTS	CLINICAL REMARKS
		Ca	P		Ca	P			
		grams	*grams*		*mgm. per cent*	*mgm. per cent*	*seconds*		
I	1	2.0	1.0		5.5	5.2	0–10	Total Prot. 8.00	Active tetany. B.P.
	2							B. M. R. +4	160/110
	3				5.76	6.4	40	Wass. neg.	Latent tetany
	4							CO – 63 V%	Improvement
	5								
	6				6.6	5.9			B.P. lower (142/92)
	7								Occas. fleeting tightness in hands
	8				6.8	7.5			
	9							Urea clearance normal	
	10								
	11				6.8	6.4	16		
	12						30		
	13				6.9	6.7			
	14								
	15								
	16								
II	17	2.0	0.27						
	18								
	19								
	20								
	21				7.4	5.5	30		
	22								
	23							CO_2 = 60	Sl. wt. loss
	24				7.3	4.3		BMR + 11	
	25						40		
	26								Distinct improvement
	27				7.5	5.5			
	28							CO_2 = 60	
	29								
	30								
	31				7.1	4.6		CO_2 = 56	
	32								
	33								
	34								
	35				7.3	4.9			

TABLE 1—*Concluded*

PERIOD	DAY AFTER ADMISSION	DAILY INTAKE		THERAPY	SERUM		TROUSSEAU	OTHER LABORATORY REPORTS	CLINICAL REMARKS
		Ca	P		Ca	P			
		grams	*grams*		*mgm. per cent*	*mgm. per cent*	*seconds*		
II	36						30		40 minutes rebreathing Trousseau 30″ start 45″ end
	37				7.4				
	38								
	39								
	40								
	41				7.1	4.9		CO_2 = 59	
III	42	2.0	0.27	Viosterol 250 D, 4 cc. daily					
	43				9.0	6.4		CO_2 = 58	Patient definitely has more subj. discomfort and nervousness. Some cramps in lt. arm
	44								
	45						30		
	46								
	47				8.0	5.5			
IV	48	2.0	0.27	Viosterol omitted			0–10		Tightness in epigastrium
	49								
	50				7.2	6.4		CO_2 = 64	Active tetany
	51								Occas. cramps in hands
V	52	2.0	0.27	Magnesium carbonate 2.1 gm. daily	6.6	6.4		CO_2 = 61	Occas. cramps in fingers
	53								
	54								
	55				6.7	5.5		CO_2 = 64	
	56								
	57								
	58				6.9	6.9		CO_2 = 54	
	59								
	60								Feeling fairly well. No active tetany
	61				7.3	8.3			
	62								
VI	63	2.0	0.27	Parathormone 80 units	7.2	7.6			
					7.1	4.5			
	64				8.8	3.3	Neg. 5 min.		
	65				9.2	3.9			
	66				9.0	4.3	Neg. 5 min.		Feeling entirely well. No cramps.

Following discharge from the metabolism ward she felt well until she became hungry upon her low phosphorus diet. She began to eat a number of foods, many of which contained phosphorus in considerable quantity. Soon thereafter there was a return of nervousness, twitching and cramps in her hands and legs. These

TABLE 2

H. C. Second admission

PERIOD	DAY AFTER ADMISSION	DAILY INTAKE		THERAPY	SERUM		TROUSSEAU	CLINICAL REMARKS
		Ca	P		Ca	P		
		grams	*grams*		*mgm. per cent*	*mgm. per cent*	*seconds*	
VII	1	2.0	0.27		6.64	6.9		
	2						30	
	3				7.0	7.1	20	
VIII	4	2.0	0.27	Viosterol 250 D, 4 cc. daily	6.9	6.6	45	
	5						30	
	6						40	
	7				6.9	5.5	45	
	8						45	
	9				7.0	7.6	35	Feeling badly,
	10						45	nervous and restless
IX	11	2.0	0.27	No viosterol	6.6	6.0	45	
	12						45	
	13						45	
X	14	2.0	0.27	Mg (OH), 2.7 gm. daily	7.0	5.9	35	Feeling better
	15						45	
	16						45	
	17				6.2	6.4	35	
XI	18	2.0	0.27	Parathormone, 80 units	6.7	6.4	25, A.M.	
							55, 6 P.M.	Very much
	19				6.5	5.1		better
	20				7.7	3.9	80	Quite relieved.
	21				9.2	4.5	90	"Ready to go home"

gradually increased in severity and she was brought back to the hospital for further study.

She was again placed on the metabolism ward. At this time she showed essentially the same findings as on the first admission. The course during the second admission is shown in table 2, and chart I.

At the time of admission on both occasions the serum inorganic phosphorus was very high, the serum calcium low. At the time of the second admission she again showed tetany bordering on the active type.

The urine showed no abnormality. The urea clearance test was normal. By x-ray her bones showed normal calcification. Eye-grounds showed some arteriosclerosis. There were a few lenticular opacities, but no definite cataracts.

Her urinary calcium and phosphorus excretion (not reported in detail in this paper) were both very low.

EXPERIMENTAL

During both admissions the patient remained on the metabolism ward of the Osler clinic. For purposes of reference, the chart and tables are divided into periods corresponding to alterations in the therapeutic régime. The general plan of treatment was as follows: First, in order to ascertain the effect of the calcium and phosphorus intake upon the levels of these constituents in the blood and upon the clinical symptoms, the patient was given a diet which contained 1.0 gm. of phosphorus and 2.0 gm. of calcium daily. Calcium lactate was added to the food to make up the required amount of calcium. This is designated as period I. Without altering the calcium intake, the phosphorus ingestion was then lowered to 0.27 gm. daily (Period II). In decreasing the phosphorus intake, the caloric content of the diet was necessarily reduced and became somewhat inadequate. As some body proteins were probably burned during the slight weight-loss which occurred, the actual intake of phosphorus was probably slightly higher. The carbon dioxide combining power of the blood was followed and showed no significant diminution (table 1).

Keeping the phosphorus and calcium intakes constant at 0.27 gm. and 2.0 gm. daily, respectively, the following therapeutic agents were then administered singly: Irradiated ergosterol, magnesium basic carbonate, and parathyroid extract. During the second admission these substances were again given in the same amount and order. The periods of therapy (except X and XI) were separated by control periods. During these periods, as during the entire second admission, the calcium and phosphorus intake were kept constant at 2.0 gm. and 0.27 gm. daily, respectively.

Finally, after the patient had been discharged the second time, the calcium intake was raised to approximately 5.0 gm. daily by giving

calcium chloride. Here again the phosphorus intake was approximately the same (0.27 gm. daily) and no other therapeutic agent was given.

The changes in treatment are clearly shown in outline in both tables and chart.

All blood samples were taken while the patient was fasting and at rest. With the exception of those in periods VI and XI in which several blood samples were taken during the day, all determinations recorded were made upon blood drawn in the morning. The sera for inorganic phosphorus and calcium were separated immediately, and the inorganic phosphorus filtrate made at once. All precautions recently stressed by Martland and Robison (4) were observed. The serum calcium was determined by the method of Kramer and Tisdall (5), the inorganic phosphorus by that of Fiske and Subbarow (6).

OBSERVATIONS

With the exception of the last period (XII), when the patient was not in the hospital, all significant observations are shown in the chart and tables. It is less confusing to follow them there and only period XII need be recounted in detail here.

Period XII. Very high calcium intake. Calcium chloride

This period covers the time following the second discharge from the hospital during which she kept to her diet but was very active. For a time she felt well, then began to have a "heaviness" in her hands and a few mild cramps. She was given calcium lactate 12 gm. daily. She returned March 25, 1932, still complaining of "heaviness" in hands and feet, but having had no actual cramps. Chvostek's sign positive. Trousseau's sign positive in 40 secs. The serum calcium was 6.6 mgm. per 100 cc., the inorganic phosphorus 6.9 mgm. per 100 cc. She was then given 24 cc. daily of a 45% solution of $CaCl_2$ (*i.e.*, 4.0 gm. Ca. daily). In two weeks she had improved very rapidly, although the calcium chloride had caused, as it often does, some epigastric burning. Chvostek's sign was only faintly positive, and the Trousseau sign was, however, negative after 5 minutes' compression. The serum calcium had risen from 6.6 to 8.5 mgm. per 100 cc., the

inorganic phosphorus had fallen from 6.9 to 4.5 mgm. per 100 cc. During this time she had continued her low phosphorus diet.

DISCUSSION

The classical findings of idiopathic hypoparathyroidism occur as well in post-operative cases. They are:

1. High serum phosphorus.
2. Low serum calcium.
3. Low urinary phosphorus excretion.
4. Low urinary calcium excretion.
5. Tetany, often exaggerated by exertion.
6. Tendency to cataract formation.
7. Normal bones by x-ray.

In hyperparathyroidism (except in the terminal stages) (7) exactly the reverse changes are found.

In the case under discussion all criteria mentioned above were present, except for cataracts. A few lenticular opacities were noted, but upon later examination, consultant oculists decided they were not cataracts.

There were a number of subjective symptoms aside from actual cramps, which were considered manifestations of tetany. "Nervousness," restlessness, and vague discomfort, tingling and numbness and stiffness in the extremities, tightness in the epigastrium were the symptoms that she described most often. The length of compression necessary to elicit the Trousseau sign is shown in the tables.

THE INFLUENCE OF THE CALCIUM AND PHOSPHORUS INTAKE

Since the work of MacCallum and Voegtlin (9), attention has been largely directed to the calcium metabolism in parathyroid deficiency. The relief of symptoms is usually attributed to an increase in the serum calcium, or, more recently, to an increase in the active fraction thereof. Evidence is now accumulating to show that, with a given calcium level below normal, the degree of tetany may depend upon other factors, one of the most important of which is the level of the inorganic phosphorus in the serum.

The present case serves to illustrate the conditions described above. In period II, with a calcium level of 7.2 and an inorganic phosphorus

of 4.9 mgm. per 100 cc. serum, there was only latent tetany, whereas in period IV with the same calcium level and an inorganic phosphorus of 6.4 mgm. per 100 cc. of serum, active tetany occurred. Greenwald (10 (11) (12) has repeatedly pointed out that in hypoparathyroidism the abnormalities of phosphorus metabolism were possibly more important than those of calcium metabolism. Other workers (1) (2) (8) have found evidence to suggest that the phosphorus changes in parathyroid deficiency are perhaps primary rather than secondary. Recently, Shelling (13), working with rats, has indicated the importance of the phosphorus intake as well as that of calcium in relation to symptoms in parathyreoprivic animals. He found that either low calcium or high phosphorus diets were conducive to tetany in rats deprived of their parathyroid glands. Low phosphorus or high calcium diets, on the other hand, tended to decrease the tetany.

Our first consideration in the treatment of this patient was to relieve her symptoms and to effect a change toward the normal in her serum calcium and phosphorus. As has been stated, the first means tried was regulation of the phosphorus and calcium intake.

In period I with a moderately high calcium intake (2.0 gm. daily) and a low phosphorus intake (1.0 gm. daily) the serum calcium rose (chart I) and the symptoms were partly relieved. She still had some tingling in her fingers and a feeling of tightness in her hands. During this time the serum phosphorus also rose to 7.5 mgm. per 100 cc. In period II with the same calcium intake, when the phosphorus intake was lowered to 0.27 gm. per diem, the serum phosphorus gradually fell until it reached a level of just under 5.0, on some days as low as 4.3 mgm. per 100 cc. The serum calcium rose to 7.2 mgm. per 100 cc., a somewhat higher level than it had reached in period I. Her symptoms were relieved. She was quite comfortable, although still in bed.

During period XII, the patient was at home and very active. The adverse effect of exercise upon tetany has been previously described (2). In the present instance under the conditions of active life, the régime of minimal phosphorus and moderately high calcium intake (2.0 gm. daily) was no longer effective in relieving her symptoms and maintaining fairly normal levels of calcium and inorganic phosphorus in the blood.

Raising the calcium intake to approximately 5.0 gm. daily by giving

calcium chloride resulted in striking improvement in her symptoms and an elevation in two weeks' time of her serum calcium from 6.6 to 8.5 mgm. per 100 cc. During the same period the serum inorganic phosphorus fell from 6.9 to 4.5 mgm. per 100 cc. The Trousseau sign, which had been positive in 40 seconds before the calcium chloride was begun, was found, after two weeks' time, to be negative (5 minutes' compression). It is to be noted that part of the striking improvement in this period was probably due to the tendency of calcium chloride to produce a slight acidosis. The beneficial results obtained, should, therefore, not be attributed entirely to the high calcium and minimal phosphorus intake.

In other cases, not studied in sufficient detail to be reported here, we have found the use of a high calcium and low phosphorus intake to be beneficial.

The series of observations just described afford a comparison of the effects upon the serum calcium and phosphorus, and upon the clinical symptoms of different ratios of calcium and phosphorus ingested. They serve to emphasize the importance of regulating both calcium and phosphorus intake in treating hypoparathyroidism.

IRRADIATED ERGOSTEROL

This substance has recently been highly recommended (15) as an agent for the alleviation of tetany in hypoparathyroidism. In regard to its activities, there are many conflicting ideas, partly owing to difference of dosage and partly as a result of failure to control other factors, such as the calcium and phosphorus intake. In the present case H. C., the use of viosterol[2] was accompanied by definite untoward effects. In period III the serum calcium at first rose and then returned to its former level. The serum inorganic phosphorus was definitely increased and active tetany supervened. In period VIII, during the second admission, the effects of viosterol upon the serum inorganic phosphorus were irregular, and the serum calcium level was not appreciably affected. Again, however, mild symptoms appeared.

On the other hand, in the recent careful observations of Bauer, Marble, and Claflin (16) upon a patient with hypoparathyroidism,

[2] Mead, Johnson and Co.

the administration of irradiated ergosterol in combination with large amounts of calcium chloride and a moderately restricted phosphorus intake resulted in a marked rise of serum calcium and fall of serum inorganic phosphorus. That this effect was achieved, at least in part, by the calcium chloride was shown by the fact that when the calcium chloride was reduced and the ergosterol continued, the reverse effects took place, *i.e.*, the serum calcium fell and the inorganic phosphorus rose.

Shelling (14) has reported that large doses of viosterol will raise the blood calcium and alleviate the symptoms of tetany in parathyreoprivic rats.

Certainly in our case the effects of viosterol were not good. It is possible that the dosage (4.0 cc. daily, viosterol 250D) was too large, or that it is necessary to use a very high calcium intake with this vitamin to obtain good results.

MAGNESIUM SALTS

The magnesium salts were given to this patient as a result of some work of Dr. Shelling of the department of pediatrics and upon his suggestion. From the present observations only one point arises for discussion at this time. During the administration of magnesium salts (period V) the serum inorganic phosphorus rose to a high level. With a serum calcium of 6.9 mgm. per 100 cc. somewhat lower, and a serum phosphorus of 6.9 mgm. per 100, definitely higher than the levels at which active tetany had occurred in the preceding period, the tetany was again latent. This suggests that possibly the magnesium may render inactive some of the blood inorganic phosphorus.

PARATHYROID EXTRACT

The administration of 80 units of parathormone was followed in both instances (periods VI and XI) by a prompt and very rapid fall of the serum inorganic phosphorus to a normal level. A few hours after the beginning of the fall in serum phosphorus, the serum calcium began to rise rapidly and in both instances, twenty-four hours after the injection, had reached a level of 9.2 mgm. per 100 cc. Upon both occasions, after the administration of parathormone the patient felt entirely well. The symptomatic improvement began within about 8 hours after the

injection. The Trousseau sign became negative in period VI, but in period XI could still be elicited by compression for 90 seconds. In both instances the site of intramuscular injection was massaged vigorously for 5 minutes. This may have contributed to the rapidity of absorption of the hormone. Concerning the mode of action of the hormone, discussion appears elsewhere (2) (8). The results from parathormone in this patient were quite different from those obtained with viosterol. The difference is most clearly shown in the effects upon the serum inorganic phosphorus and in the effect upon the clinical symptoms.

SUMMARY AND CONCLUSIONS

1. A case of postoperative hypoparathyroidism is reported.

2. A calcium intake of 2.0 gm. per diem, and a limitation of the phosphorus intake to 1.0 gm. per diem, resulted in partial relief of symptoms and a rise of serum calcium. The serum phosphorus also rose. Further limitation of the phosphorus intake to 0.27 gm. daily resulted in further relief of symptoms, slightly higher rise in serum calcium and a fall of serum phosphorus toward normal. Finally, when the patient was at home and exercising, the above régime was ineffective. Raising the calcium ingestion to 5.0 gm. daily by giving calcium chloride resulted in a return toward normal of serum calcium, a fall toward normal of serum phosphorus and relief of symptoms. In other cases, in which similar ratios of phosphorus to calcium ingested have been tried, beneficial results have been obtained. Emphasis is placed upon the regulation of both calcium and phosphorus intake when treating hypoparathyroidism.

3. The administration of viosterol was followed in this patient by precipitation of active tetany.

4. Observations were made upon the effect of administration of a magnesium salt.

5. Parathormone injection resulted in a very rapid fall of serum phosphorus, rise of serum calcium and symptomatic improvement.

The author wishes to thank Miss M. Struve for her careful supervision of the dietary régime and for preparing the chart.

BIBLIOGRAPHY

(1) ALBRIGHT, F., BAUER, W., ROPES, M., and J. C. AUB: *J. Clin Invest.*, 1929, vii, 139. Studies in Calcium and Phosphorus Metabolism. IV. The Effect of the Parathyroid Hormone.

(2) ALBRIGHT, F., AND R. ELLSWORTH: *ibid.*, 1929, vii, 183. Studies on the Physiology of the Parathyroid Glands. I. Calcium and Phosphorus Studies on a Case of Idiopathic Hypoparathyroidism.

(3) ALBRIGHT, F., BAUER, W., COCKRILL, AND R. ELLSWORTH: *ibid.*, 1931, ix, 659. Studies in Physiology of the Parathyroid Glands. II. The Relation of the Serum Calcium to the Serum Phosphorus at Various Levels of Parathyroid Activity.

(4) MARTLAND, M., AND R. ROBISON: *Biochem. J.*, 1924, xviii, 765. Note on the Estimation of Phosphorus in Blood.

(5) KRAMER, B., AND F. F. TISDALL: *J. Biol. Chem.*, 1921, xlviii, 223. The Direct Determination of Sodium, Potassium, Calcium and Magnesium in Small Amounts of Blood.

(6) FISKE, C. H., AND Y. SUBBAROW: *ibid.*, 1925, lxvi, 375. The Colorimetric Determination of Phosphorus.

(7) ALBRIGHT, F., BAUER, W., CLAFLIN, F., AND J. R. COCKRILL: Studies in Parathyroid Physiology. III. The Effect of Phosphate Ingestion in Clinical Hyperparathyroidism.

(8) ELLSWORTH, R.: Studies in Physiology of Parathyroid Glands. V. The Action of Parathyroid Extract on the Renal Threshold for Phosphorus. *J. Clin. Invest.*, 1932, xi, 1011.

(9) MACCALLUM, W. G., AND VOEGTLIN: *Johns Hopkins Hosp. Bull.*, 1908, xix, 91. On the Relation of the Parathyroid to Calcium Metabolism and the Nature of Tetany.

Idem: *J. Exp. Med.*, 1909, xi, 118. The Relation of Tetany to the Parathyroid Glands and to Calcium Metabolism.

(10) GREENWALD, I.: *J. Biol. Chem.*, 1926, lxvii, 1. The Effects of the Administration of Calcium Salts and of Sodium Phosphate upon the Calcium and Phosphorus Metabolism of Thyroparathyroidectomized Dogs, with a Consideration of the Nature of the Calcium Compounds of Blood and their Relation to the Pathogenesis of Tetany.

(11) *Idem*: *ibid.*, 1924, lix, 329. Are Guanidines Present in the Urines of Parathyroidectomized Dogs?

(12) *Idem: ibid.*, 1924, lxi, 649. Some Chemical Changes in the Blood of Dogs after Thyroparathyroidectomy.

(13) SHELLING, D. H. *ibid.*, 1932, xcvi, 195. Calcium and Phosphorus Studies. I. The Effect of Calcium and Phosphorus of the Diet on Tetany, Serum Calcium and Food Intake of Parathyroidectomized Rats.

(14) *Idem: ibid.*, 1932, xcvi, 215. II. Effect of Diet and Viosterol on the Tetany and on the Serum Calcium of Parathyroidectomized Rats.

(15) BOOTHBY, W. M., HAINES, S. F., AND I. DE J. PEMBERTON: *Am. J. Med. Sc.*, 1931, clxxxi, 81. Post-operative Parathyroid Insufficiency.

(16) BAUER, W., MARBLE, A., AND F. CLAFLIN: *J. Clin. Invest.*, 1932, xi, 47. Studies on the mode of Action of Irradiated Ergosterol. IV. In Hypoparathyroidism.

STUDIES IN EXPERIMENTAL SYPHILIS

X. Observations on Cross-inoculations with Heterologous Strains of Syphilitic Virus[1,2]

ALAN M. CHESNEY, M.D., THOMAS B. TURNER, M.D. and FRANKLIN H. GRAUER, M.D.

From the Syphilis Division of the Department of Medicine of the Johns Hopkins University

It has been definitely established that the resistance which develops in rabbits during the course of an experimental syphilitic infection is largely, if not entirely, strain-specific and not species-specific. Rabbits infected with one strain of *T. pallidum* and subsequently treated can often be infected later with another strain of this same microorganism, while retaining their resistance against the strain with which they were originally infected. There is general agreement on this point by those investigators who have busied themselves with the problem.

It is by no means certain that the same condition holds true for man. Indeed, Kolle (1) interprets the comparative scarcity of reinfections with syphilis, even in countries where treatment facilities are good and cases are energetically treated, as indicating that in man infection with one strain of *T. pallidum* leads to a resistance which is operative against other strains of this organism as well as against the homologous one. Thus, he says that in the rabbit there develops what he calls a "mono-immunity," an immunity against the homologous strain only, while in man, on the other hand, there develops a "pan-immunity" or immunity against many strains. It is more than likely that his interpretation is the correct one.

However, it must be admitted that the customary methods of testing the resistance of syphilitic rabbits, that is, by the intratesticular or intradermal injection of emulsions of syphilitic virus, or by implanting bits of syphilitic tissue in the subscrotal sac, are at best crude methods

[1] Aided in part by a grant from the Committee on Research in Syphilis, Inc., and from the American Medical Association.

[2] Submitted for publication October 15, 1932.

of inoculation when compared with the usual mode of transmission of the disease in man. The former are certainly calculated to favor the inciting agent rather than the host. Because of this fact, it was thought advisable to conduct a series of cross-inoculation experiments in rabbits in which the method of inoculation, when testing resistance against heterologous strains, approximated more closely the usual mode of transmission of the disease in man. The particular method of inoculation selected for testing the resistance of the rabbit consisted in the deposition of active heterologous syphilitic virus upon a mucous membrane surface which was intact so far as inspection with the naked eye could determine. It has been definitely established that normal rabbits can be infected with syphilis in this manner (2, 3). As will be seen from the protocols of the experiments, the resistance which develops in syphilitic rabbits is not proof against subsequent inoculation with heterologous strains even when the test inoculations are carried out by placing the virus upon what appears to be an intact mucous membrane surface.

EXPERIMENTAL

Two series of experiments were carried out on different series of animals and at different seasons of the year, so as to have one set of test inoculations performed in the early winter months, another in the spring months. All of the test animals were originally inoculated intratesticularly with the Nichols strain of *T. pallidum* and were treated with intravenous injections of arsphenamine, each animal receiving six doses of 20 mg. per kg. each, at intervals of one week. Treatment was begun at a time when the immunity against homologous strains is known to be well developed in rabbits, in this case six months or longer following the original infection.

From each of the treated animals a single popliteal node was removed, emulsified in salt solution and the resultant emulsion injected intratesticularly into each of two normal rabbits. This node transfer was carried out 67 to 127 days after the last injection of arsphenamine, and served as a measure of the efficacy of treatment.

Test inoculations were then made with testicular emulsions of a heterologous strain of *T. pallidum*, designated Strain F, which was isolated in this laboratory approximately 9 years ago and with which

other cross-inoculation experiments have been performed. We knew from previous experience that infection with the Nichols strain of *T. pallidum* did not protect rabbits against this strain (F) if it were inoculated intratesticularly (4).

The test inoculations were made by dropping a measured amount of virus suspension, 0.05 cc., into the pouch formed by drawing the prepuce forward. After the virus was deposited the prepuce was held in the forward position for a full minute, measured by the watch, before releasing it. The mucous membrane of the glans penis and of the prepuce appeared normal in every instance at the time of inoculation. There was not the slightest sign of infection with *T. cuniculi* in any of the animals, and indeed our rabbit stock has been singularly free from this infection.

The test animals were observed at frequent intervals for a period of 120 days or longer following the test inoculation, except in instances where the animals died prematurely. From 119 to 132 days after reinoculation the remaining popliteal node was removed from each of the animals in which no lesion or a doubtful lesion had developed at the site of inoculation, an emulsion of the node was prepared and inoculated intratesticularly into each of two normal rabbits. The test animals were then discarded. During the period of observation following the test inoculation, Wassermann reactions were carried out on the serum of all the test rabbits according to the technic which we have been accustomed to use in rabbits (5).

Controls

As a control on the manner of inoculation two series of immune rabbits were inoculated by a different method; one by injecting the virus just beneath the epithelial layer of the prepuce, the other by injecting the virus intratesticularly in the usual manner. The virulence of the virus was controlled by the inoculation of series of normal rabbits by the methods of inoculation employed in the case of the test animals, namely, (1) deposition of the virus on the intact mucous membrane surface of the prepuce, (2) injection of the virus beneath the epithelial layer of the prepuce and (3) intratesticular injection. Lymph node transfers were performed on all control animals in which no lesions or doubtful lesions only appeared at the site of inoculation.

TABLE I

Experiment 1

	RABBIT NUMBER	FIRST INFECTION				SECOND INFECTION									
			Node transfer										Node transfer		
		Day of disease treatment began	Day of disease	Days after treatment ended	Result	Day of disease reinoculated	Days after treatment ended	Local lesion and incubation period	Dark field examination	Enlargement of regional lymph node	Generalised lesions	Wassermann reaction	Days after reinoculation	Result	Final result of reinoculation
Group A. Reinoculated by dropping virus in prepucial sac	2504	261	363	67	Negative	533	237	+ (30)	+	+	0	0	Not necessary		Positive
	2507	261	363	67	Negative	533	237	+ (30)	+	+	Died under anesthesia				Positive
	2509	261	363	67	Negative	533	237	0	−		Found dead 26 days after reinoculation				Unknown
	2512	261	363	67	Negative	533	237	? (51)	−	+	0	0	125	+	Positive
	2517	261	363	67	Negative	533	237	? (37)	−	?	0	0	125	0	Negative
	2524	261	365	69	Negative	533	237	0	−	+	0	0	131	0	Negative
	2581	181	285	69	Negative	446	230	?	−	+	0	0	131	+	Positive
	2582	181	285	69	Negative	446	230	0	−	0	0	0	131	0	Negative
Group B. Reinoculation by submucous injection	2586	181	285	69	Negative	446	230	+ (15)	+	+	0	0	Not necessary		Positive
	2587	181	285	69	Negative	446	230	+ (23)	+	+	0	?	Not necessary		Positive
	2588	181	285	69	Negative	446	230	+ (64)	+	+	+ (S)	0	Not necessary		Positive
	2589	181	285	69	Negative	446	230	+ (15)	0	0	+ (S)	0	Not necessary		Positive
Group C. Reinoculation by intratesticular injection	2591	181	291	75	Negative	446	230	+ (58)	+	0	0	0	Not necessary		Positive
	2592	181	291	75	Negative	446	230	+ (12)	+	0	0	0	Not necessary		Positive
	2593	181	292	76	Negative	446	230	0	−	0	0	0		+	Positive
	2594	181	291	75	Negative	446	230	+ (32)	+	+	0	0	Not done		Positive

Controls

Group A. Controls inoculated by dropping virus in prepucial sac	3188							? (37)	−	+	0	0	131	+	Positive
	3190							+ (47)	+	?	+ (B.E.)	0	Not necessary		Positive
	3191							?	−	?	0	0	131	0	Negative
	3192							0	−	?	0	0	131	0	Negative
	3193							+ (40)	0	+	0	0	131	+	Positive
	3194							+ (26)	+	+	+ (B.S.)	0	Not necessary		Positive
	3195							? (23)	−	?	0	0	132	0	Negative
	3197							+ (33)	0	+	+ (S)	0	Not necessary		Positive
Group B. Controls inoculated by submucous injection	3198							+ (12)	−	+	0	0	132	+	Positive
	3199							+ (15)	+	+	+ (S.B.)	?	Not necessary		Positive
	3201							+ (12)	0	+	+ (T)	0	Not necessary		Positive
	3202							+ (12)	+	+	+ (B.S.T.)	+	Not necessary		Positive
Group C. Controls inoculated by intratesticular injection	3203							+ (12)	+	+	+ (T.B.)	+	Not necessary		Positive
	3204							+ (12)	+	+	+ (T.E.)	+	Not necessary		Positive

Key to abbreviations under "Generalised Lesions."

S = Skin
B = Periosteal bone
T = Testicle
E = Eye

TABLE II

Experiment 2

	Rabbit number	First infection				Second infection									
		Day of disease treatment began	Node transfer: Day of disease	Node transfer: Days after treatment ended	Node transfer: Result	Day of disease reinoculated	Days after treatment ended	Local lesion and incubation period	Dark field examination	Enlargement of regional lymph node	Generalised lesions	Wassermann reaction	Node transfer: Days after reinoculation	Node transfer: Result	Final result of reinoculation
Group A. Reinoculated by dropping virus in preputial sac	2620	179	293	79	Negative	512	298	? (44)	−	+	0	0	119	0	Negative
	2625	179	296	82	Negative	512	298	? (64)	−	+	0	0	119	0	Negative
	2626	179	296	82	Negative	512	298	? (99)	−	?	0	0	119	0	Negative
	2627	179	296	82	Negative	512	298	+ (49)	+	+	0	?	Not necessary		Positive
	2630	179	299	85	Negative	512	298	? (71)	−	+	0	0	119	0	Negative
	2648	179	309	95	Negative	512	298	+ (44)	+	+	0	0	Not necessary		Positive
	2598	181	298	82	Negative	536	320	? (47)	−	0	0	0	119	+	Positive
Group B. Reinoculated by submucous injection	2650	184	305	86	Negative	508	289	+ (16)	+	Animal died		19th day			Positive
	2651	184	306	87	Negative	508	289	+ (11)	+	+	0	0	Not necessary		Positive
	2652	184	306	87	Negative	508	289	+ (8)	0	?	0	0	120	+	Positive
	2658	184	306	87	Negative	508	289	+ (16)	+	?	0	0	Not necessary		Positive
Group C. Reinoculated by intratesticular injection	2654	184	306	87	Negative	508	289	0	−	−	0	0	120	+	Positive
	2686	184	297	78	Negative	494	275	+ (11)	+	0	0	+	Not necessary		Positive
	2687	184	346	127	Negative	494	275	0	0	0	0	0	120	+	Positive
	2688	184	346	127	Negative	494	275	? (16)	0	?	0	0	120	+	Positive

Controls

| | | |

RESULTS

The pertinent data concerning the two experiments and the results obtained are shown in Tables I and II. Only those results are recorded as finally positive in which (1) *T. pallidum* could be demonstrated in the lesion at the site of inoculation, or (2) in those instances in which the popliteal node transfer after reinoculation was positive, or (3) in those instances in which characteristic generalised lesions involving testis, skin, bone or eyes were observed.

The results of both experiments were essentially the same and are summarised in Table III.

Inspection of Table III shows that reinoculation of immune rabbits with heterologous strains by depositing the virus on the intact mucous membrane of the preputial sac led to a second infection in almost the same percentage of test animals as in the control normal animals. Reinoculation by the submucous injection led to infection in 100 per cent of the test animals, and the same held true when reinoculation was carried out by intratesticular injection.

It should be noted that in the control normal animals inoculated by depositing the virus in the preputial sac, there were 3 symptomless infections in a total of 10 positive results, or 30 per cent, whereas in the "immune" group inoculated in a similar manner the incidence of such lesions was 3 in a total of 7 positive results, or 43 per cent. In our opinion this difference between the two groups in the matter of incidence of symptomless infections is so slight as to be insignificant, and is not to be interpreted as indicating any appreciable resistance against heterologous strains.

The lesions which were observed in both the test and the control animals inoculated by dropping the virus in the preputial sac were much milder and slower in appearing than the lesions developing after submucous inoculation. In the case of the latter, characteristic chancres frequently occurred, but the deposition of the virus on the intact mucous membrane was frequently followed by a transitory localized erythema or slight area of induration which might easily have been missed had the observer not been particularly on the lookout for the occurrence of a lesion. Dark field examinations of serum from any of these suspicious lesions were purposely not carried out, for fear lest the trauma incidental to such procedures would obscure

the results of the experiment. However, the comparatively long incubation period and the occurrence of enlargement of the regional lymph node suggested that these lesions were syphilitic and the positive lymph node transfer obtained in many instances left little doubt that they were in reality of syphilitic origin. There were a few instances in which suspicious lesions developed and were accompanied by enlargement of the regional lymph node but in which the popliteal node transfer proved to be negative. It would not be surprising if some of these cases were actually instances of successful reinoculation, but they were not proved to be such and for that reason are recorded as negative.

TABLE III

Summary of experiments 1 and 2

METHOD OF INOCULATION	TOTAL	POSITIVE	NEGATIVE	DOUBTFUL	PER CENT POSITIVE
Deposition of virus on mucous membrane:					
"Immune" animals	15	7	7	1	46
Normal animals	16	10	6	0	62
Submucous injection:					
"Immune" animals	8	8	0	0	100
Normal animals	8	7	0	1	87
Intratesticular injection:					
"Immune" animals	8	8	0	0	100
Normal animals	4	4	0	0	100

DISCUSSION

The results of the foregoing experiments are sufficiently clear cut to admit of but one interpretation, namely, that the specific resistance which develops in syphilitic rabbits during the course of their infection is not effective against heterologous strains of *T. pallidum* if the method of testing this resistance consists in depositing the virus emulsion upon the intact genital mucous membrane. Certainly such a method of reinoculation may be regarded as favoring the host rather than the inciting agent, nevertheless, even under these conditions the immunity must be regarded as strain-specific, rather than species-specific.

It is not at all certain, however, that the same state of affairs holds

true in the case of man. Kolle called attention to this point several years ago, and our own experience with syphilitic reinfection in man bears out his contention that in human beings acquired immunity to syphilis is probably species-specific and not strain-specific. In an analysis by one of us (Turner) of ten thousand consecutive cases of syphilis admitted to the Syphilis Clinic of the Johns Hopkins Hospital (6) there were encountered 34 cases which were diagnosed as probable reinfections. Admitting that all of these were undoubted cases of reinfections, the resulting incidence of 0.34 per cent is an admittedly low figure. Moreover, as Halley and Wassermann (7) brought out in their statistical study of the cases of reinfection reported in the literature, the overwhelming majority (97.8 per cent) of all cases of reinfection occurred in individuals who came under treatment for their first infection during the primary or secondary stage of their disease, and in only 1.6 per cent of their cases was there evidence that the patient had entered the late period of the disease before receiving treatment.

In our experiments, however, the rabbits had passed through the early stages of the infection and had entered the period of latency before treatment was begun. Yet they proved to be susceptible to a second infection with a heterologous strain of syphilitic virus, deposited on an intact genital mucous membrane. When one compares the results obtained in these animals with what one observed in man, it is difficult to escape the conclusion that the immunologic relationships in the two species are different in so far as syphilis is concerned. The comparative scarcity, in human beings, of second infections with syphilis, can scarcely be explained on the assumption that individuals who have acquired syphilis and have been treated for it do not acquire a second infection because they are not exposed thereafter. At any rate such an assumption for the 10,000 cases of syphilis referred to above seems scarcely justified, for of the 10,000 cases analysed 6,562 occurred in negroes, and it is universally admitted that in members of this racial group in the United States exposure to syphilis occurs much more frequently than in whites.

The facts brought out by the experiments reported in this paper, and by the study of a large group of patients with syphilis, already alluded to, furnish additional support for the view advanced by Kolle

that the immunity to syphilis acquired by rabbits is limited to homologous strains of the syphilitic virus, but in man may not be so limited but may be effective against other strains as well. The reasons for this difference in the behavior of the two species are not apparent at the present time.

SUMMARY AND CONCLUSIONS

Rabbits infected with one strain of *T. pallidum* (Nichols strain) and treated comparatively late in the course of their disease, *i.e.*, 6 to $8\frac{2}{3}$ months after the first inoculation, were subsequently inoculated with a different strain of *T. pallidum* (Strain F) by depositing the virus on the intact genital mucous membrane. Under these conditions 46 per cent of the test animals were successfully infected a second time. In an equal number of control normal animals inoculated in a similar manner, the incidence of positive inoculations was 62 per cent. The conclusion is drawn that the immunity which develops in rabbits during the course of a syphilitic infection is strain-specific even when tested as outlined above, that is to say, in a manner which favors the host rather than the inciting agent.

The occurrence of syphilitic reinfection in man and its bearing upon the reported experiments with rabbits is discussed.

BIBLIOGRAPHY

(1) KOLLE: *Deutsche. Med. Wchnschr.*, 1926, lii, 11.
(2) REASONER, M. A.: *Am. J. Syph.*, 1917, i, 478.
(3) BROWN, W. H. AND L. PEARCE: *J. Exper. Med.*, 1924, xxxix, 645.
(4) CHESNEY, A. M., HALLEY, C. R. L., AND J. E. KEMP: *J. Exper. Med.*, 1927, xlvi, 223.
(5) KEMP, J. E., CHESNEY, A. M., AND A. K. POOLE: *Bull. Johns Hopkins Hospital*, 1926, xxxix, 132.
(6) TURNER, T. B.: *ibid.*, 1930, xlvi, 159.
(7) HALLEY, C. R. L. AND H. WASSERMANN: *Arch. Int. Med.*, 1928, xli, 843.

HYPERPROTEINEMIA ASSOCIATED WITH MULTIPLE MYELOMA[1]

With Report of a Case in Which an Extraordinary Hyperproteinemia Was Associated with Thrombosis of the Retinal Veins and Symptoms Suggesting Raynaud's Disease[1]

MAXWELL M. WINTROBE, M.D., Ph.D., and MARY V. BUELL, Ph.D.

(From the Medical Clinic, The Johns Hopkins University and Hospital, Baltimore)

The occurrence of abnormal protein constituents in the blood in cases of multiple myeloma is in itself sufficiently unusual to merit attention. Although Ellinger (1), in 1899, had reported the presence of abnormal protein in the blood of a patient suffering from multiple myeloma, no other report appeared until 1917, when Jacobson (2) described the occurrence of a heavy creamy precipitation during the inactivation at 60°C. of the blood serum of his patient. A similar experience during the performance of the Wassermann test has been recorded by Short and Crawford (3). Abderhalden (4) in 1919 demonstrated, in one case, the presence of a protein in the plasma which resembled Bence-Jones protein; Gabbe (5) and Zadek and Lichtenstein (6) have made similar observations. Perlzweig *et al.* (7) and Johansen (8) reported a striking hyperproteinemia in one case each. In view of the fact that Bence-Jones protein is found in the urine in at least 65 per cent of all cases of multiple myeloma, the occurrence of discovered chemical abnormalities in the circulating blood is thus, in comparison, very unusual (8 instances in approximately 500 case reports).[2]

The findings in our patient are the more remarkable because disturbances in the circulation in the form of thrombosis of the retinal veins, and symptoms which first led to a diagnosis of Raynaud's

[1] Submitted for publication July 29, 1932.

[2] H. A. Reimann has recently reported another case in which chemical abnormalities were discovered in the circulating blood. (Hyperproteinemia as a cause of autohemagglutination. Observations in a case of myeloma. Jour. Am. Med. Assn., **99**, 1411–1414 (October 22), 1932.)

disease, were associated with the presence in the blood of a protein which was never found in solution in the blood, even when precautions were taken to collect and maintain the blood at body temperature. What the physical state of this protein was, within the body, was not discovered; and it is impossible to say whether it was in any way related to the unusual symptoms which the patient manifested. It is no doubt venturesome to suggest that the unusual symptoms and the extraordinary constitution of the blood were related. It can only be said that we have not been able to find in the literature a report of the occurrence of such a remarkable substance in the blood, or of any case of multiple myeloma in which disturbances of circulation suggesting Raynaud's disease were present. Thrombosis is also an unusual occurrence in multiple myeloma. The only reports are those of Wallgren (9) and of Venturi (10), each of whom described one case in which tumors in the skull gave rise to thrombosis in the intracranial sinuses. In Venturi's case there was also thrombosis of the central artery of the retina, but there is no mention of any abnormal constituent in the blood.

M. S. R. (U40905), a white housewife, 56 years of age, was admitted to the Johns Hopkins Hospital December 29, 1931, because of severe pain in the right shoulder, coldness of the hands and feet, and shortness of breath.

Her mother and sister had died of pulmonary tuberculosis. The patient herself had always been very well, however, with the exception of frequent attacks of headache. In August, 1930, she had an attack of "shingles" and since that time, according to her family physician, she had not been entirely well. In January, 1931, sharp pain commenced in the right side of the neck. This pain was very severe, radiated down to the right shoulder, would last a half to one hour, and recurred every few days. About a week after the pain in the neck began, severe burning pain in the right lumbar region, which extended around the right side of the body, appeared; and this was followed in a few days by Herpes Zoster in the same location. This disappeared in a few weeks, but the patient became increasingly irritable, excitable and emotional; she lost her appetite, and complained of nausea and "gas." In the summer of 1931, pain in the left side of the face and left eye commenced. This was thought to be due to sinusitis. The headaches at this time became more frequent. They were located chiefly in the frontal region and radiated to the zygomata. Nausea and occasionally vomiting accompanied them. About this time a peculiar mottling of the arms and lower limbs, and blueness of the fingers, toes, rims of the ears and tip of the nose, were first noticed.

In August 1931 new symptoms, coldness and blanching of the hands, feet and

even of the tongue, appeared rather suddenly. These symptoms were aggravated by cold or damp. During all this time there were intermittent exacerbations of the pain in the right shoulder, as well as attacks of pain in the right side and hip. In September, 1931, attacks of nose-bleed commenced, and recurred at about weekly intervals. The amount of blood lost was not very great. Some shortness of breath and palpitation of the heart on exertion were first noticed at this time. The blood count showed 4,200,000 red corpuscles, 80 per cent hemoglobin and 5,000 leucocytes.

In November, 1931, the patient was examined at the Union Memorial Hospital. A competent neurologist thought that she was suffering from typical Raynaud's disease. At this time very extensive eye-ground changes, which were interpreted to indicate bilateral thrombosis of the central veins of the retina, were also discovered. The blood examination showed 3,670,000 R.B.C., 70 per cent hemoglobin, 3,550 W.B.C. Kidney function was good, the phenolsulphonephthalein excretion being 70 per cent. The pains were thought to be due to bursitis or arthritis, although roentgen rays of the hip and right shoulder revealed nothing suggestive of arthritis.

After leaving the hospital the patient had an attack of severe pain on urination which was accompanied by hematuria. These symptoms were thought to be due to the presence of a renal stone. Her vision from this time on became more and more impaired; she became progressively weaker, increasingly dyspneic and lost 50 pounds in weight. The attacks of numbness and blanching of the extremities still persisting, the patient appeared at the Johns Hopkins Hospital for the cervical sympathectomy which had previously been advised.

Although she was at first admitted to the Surgical and Neuro-Surgical Services, the dyspnea, fatigue, epistaxis and prostration had by this time become so prominent and so greatly overshadowed the symptoms which had suggested Raynaud's disease, that the patient was transferred to the Medical Service for study.

Physical examination revealed a woman of large, bony framework which somewhat suggested the acromegalic type. She had apparently lost some weight, although there was still a considerable amount of subcutaneous adipose tissue. She was at times irritable, and then again apathetic. Blood crusts were present about the nose and lips. The latter, as well as the tongue and gums, were cracked and blood slowly oozed from them. The skin, especially over the face and extremities, showed blanched areas interlaced with reddish, livid blotches. This blotching became more marked on the application of cold, and tended to disappear on the application of warmth. The extremities were always warm, however, and arterial pulsation was normal. Scratching of the skin was not succeeded by erythema or by erythema and anemia. There were several bruises over the extremities and abdomen, and a few small petechial spots were found on the left arm where a tourniquet test had been made. The eyes were normally prominent, reacted normally to light and accommodation, and moved normally. The eye-grounds showed extensive hemorrhages with extreme dilatation of the veins,

findings which were interpreted as indicating thrombosis of the central veins of the retinae. The arteries were slightly smaller than normal but definitely patent. These changes were much more marked in the right eye. The mucous membranes did not look particularly pale. The teeth had all been extracted. There was no enlargement of the superficial lymph nodes.

The right shoulder was held motionless. It was swollen and extremely tender to touch. The slightest movement caused excruciating pain. There was no redness, heat or ecchymosis. Over the lungs the percussion note was everywhere resonant, the breath sounds vesicular, and at the bases there were many coarse moist râles. There was no enlargement of the heart, but a soft systolic murmur was heard at the apex. The pulse was regular and equal at the two wrists; the blood pressure was 170/100. There was moderate sclerosis of the peripheral vessels. The abdomen was held rather firmly, but there was no muscle spasm or tenderness. The firm edge of the liver was felt 2 cm. below the costal margin. The edge of the spleen was readily felt 3 cm. below the costal margin. Several examiners thought that they could feel the right kidney. Pelvic examination showed no evidence of gross disease. There was slight pitting edema over the extremities. Neurological examination was negative.

In the hospital slight fever (100 to 102°F., rectal) was noted. The blood pressure fell after admission to normal limits (115 to 130 systolic, 70 to 80 diastolic). A Queckenstedt test to determine the presence of increased intracranial pressure was negative. Examination of the urine revealed no abnormalities, except rare red cells and an occasional hyaline cast. There was no albumin in the urine, but occasionally reduction of Benedict's solution occurred. The specific gravity ranged from 1.011 to 1.021. The stools consistently gave a strongly positive benzidine reaction. The blood culture showed no growth. The Wassermann and flocculation tests were negative. The blood sugar, non-protein nitrogen, cholesterol, calcium and phosphorus were all within normal range.

Because of the bleeding a complete blood study was made. There were 3,290,000 red cells, 11.4 grams (79 per cent) hemoglobin and 31.5 cc. of packed red cells per 100 cc. of blood. The erythrocytes were somewhat larger than normal, the mean corpuscular volume being 96 c. μ but there was no distinct aniso- or poikilocytosis. Their hemoglobin content was proportionately increased. Leucocytes numbered 3,500 per c.mm. with 1 per cent myelocytes, 4 per cent juvenile neutrophiles, 69 per cent segmented neutrophiles, 24 per cent adult lymphocytes, and 2 per cent monocytes. Platelets numbered 146,000 per c.mm. Bleeding time, clot retraction time and coagulation time were normal.

In the hematocrit, the examiner was surprised to find, above the layer of packed red cells, a dense yellowish gray layer of material, 1 cm. in height, and thus making up 10 per cent of all the blood. This layer somewhat resembled the "buffy coat," consisting of packed leucocytes, which is seen in instances of marked leucocytosis or leukemia (11); but it was already known that in this case there was leucopenia instead of striking leucocytosis. The material was removed and was found to be

very viscid, smearing only with great difficulty. Microscopically, it was found to consist of brownish masses varying greatly in size.

Another hematocrit was filled with this blood, centrifuged, and placed in the icebox for further examination of the material the next day. To our surprise we observed, above the yellowish gray substance already mentioned, a cloudy white material which was suspended throughout the supernatant blood plasma. Most amazing of all was the discovery that this cloudy material disappeared at room temperature in 10 to 15 minutes, leaving the yellowish gray material as the only abnormal gross constituent of the blood. The cloudy material could be brought out at will by placing the hematocrit in the icebox, and it disappeared at room temperature in the same manner each time.

The myelophthisic blood picture (the anemia, leucopenia due largely to granulocytopenia, and the thrombocytopenia), together with the peculiar material in the hematocrit, some of which appeared and disappeared with changes in temperature, led to the suggestion that this patient was suffering from a bone marrow tumor, possibly multiple myeloma.

At this time, roentgen-ray examination of the right shoulder revealed a pathological fracture of the surgical neck of the humerus, with numerous areas of lesser density in the scapula, "probably due to metastatic bone tumor." Two days later, metastases were reported in the bones of the skull, and possibly in the right ilium. The ribs and spine were negative. By the roentgen ray it was thought that the left kidney shadow was enlarged.

Bence-Jones protein was repeatedly looked for in the urine but was not found. No significant changes occurred in the patient's condition during her stay in the hospital except that the bleeding time became prolonged, the clot did not retract satisfactorily, and the platelets dropped to 108,000 per c.mm., although the anemia did not become more severe. She left February 6, 1932, to go to her home outside of Baltimore.

For a brief period after her discharge, the patient improved, but this was soon followed by a fatal relapse. Death occurred on March 17, 1932. A synopsis of the autopsy report (J. H. H. No. 12497) follows:

There was a pathological fracture of the right humerus about 12 mm. from the head. The humerus showed an invasive kind of tumor which extended here and there in streaks and patches throughout the marrow. A good deal of myeloid tissue was left, but there were also patches of more homogeneous tumor cells which were closely bound together. The same tissue was found in the second lumbar vertebra, and there it seemed in places to push aside the trabeculae and to be extremely rich and vascularized. The bone marrow of the vertebrae was red (Dr. W. G. MacCallum).

Portions of the occipital scalp seemed to bulge over soft thickenings, but permission to examine the head could not be obtained. The mediastinal lymph nodes were greatly enlarged. A lymph node on the right bronchus was caseous, and showed some calcification.

The spleen was greatly enlarged and weighed 625 grams. The trabeculae, Malpighian bodies, and blood vessels were prominent. There were a number of calcified nodules. The liver weighed 2,100 grams and showed no gross lesions. The left kidney weighed 300 grams and the right kidney, 250 grams. There was nothing to suggest kidney damage. The other tissues examined, including the heart, great vessels and adrenals, were essentially normal.

The final anatomic diagnosis was *plasma-cell myeloma* invading the humerus and lumbar vertebra; pathologic fracture of the humerus; enlarged spleen with calcified nodules.

CHEMICAL OBSERVATIONS

Qualitative analysis of the curious substance discovered in the blood of this patient could be carried out readily enough; for it invariably precipitated spontaneously. It was washed exhaustively with water, and was then treated with successive portions of aqueous alcohol of increasing alcohol content, and finally with absolute alcohol, and then with ether. By drying in a vacuum desiccator over sulfuric acid, a white powder was obtained which went readily into solution in dilute acids or alkalies. In such solutions a precipitate was formed on neutralization. In the faintly acid solution, when heated, coagulation occurred at a low temperature (about 40°C.), but the coagulum did not go back into solution again on boiling. No tests were made to determine the effect on its solubility at 100°C. of the addition of electrolytes or other compounds.

The purified preparation responded characteristically to the usual protein color reactions. Qualitative tests were obtained for phosphorus and for sulfur. Under conditions favorable for crystallization a definite microscopic needle form was observed.

A solution of the protein in alkali gelatinized. Since this property brought to mind the nucleoproteins, several attempts were made to hydrolize the protein in such a way as to make possible the detection of a purine group if such existed. Results were negative.

In carrying out quantitative estimations of the proteins in the plasma, difficulty was encountered in obtaining an appropriate sample. In spite of the signs of disturbed circulation it seemed unlikely that such large amounts of solid substance, as were observed *in vitro*, were present *in vivo*; and, consequently, it was expected that by special care in drawing the blood the abnormal substance might be

obtained in solution. Two causes were considered as possible factors in bringing about the precipitation of this substance; namely, changes in temperature and alterations in hydrogen-ion concentration. It had been noted in the hematocrit that, when placed in the ice-box, a further small additional precipitation occurred. The possible influence of hydrogen-ion concentration was suggested by the fact that the precipitated substance, insoluble in plasma, went readily into solution in dilute acid or alkali. Consequently, blood was drawn with precautions to prevent changes in temperature and also loss of CO_2; nevertheless, the usual precipitate was found.

Obviously it was impossible to take representative samples of this plasma, in the usual way, for the determination of total protein. To overcome this difficulty, a few cubic centimeters of whole blood were drawn into a graduated centrifuge tube, and, after centrifugation, the whole plasma layer, including the precipitated substance, was separated mechanically, and was Kjeldahlized. By this procedure the total plasma protein was found to be 11.9 grams per cent, a figure which must be regarded as a minimum because it was impossible to separate all of the precipitate from the red cells. The protein content of the plasma, exclusive of the precipitated protein, was 4.65 grams per 100 c.c. If the volume of the precipitate is disregarded in the calculation, there were thus 7.25 grams of precipitated protein per 100 c.c. plasma. Again this must be regarded as a minimum value because this experiment was carried out at room temperature. As had been previously demonstrated, certain small quantities of this substance remained in solution at room temperature, which were precipitated at ice-box temperature.

Of the 4.65 grams of protein per 100 c.c. of clear plasma, 2.50 grams were found to be albumin as determined by macrotechnique. Thus the albumin to globulin ratio in this fluid was 54 to 46, an approximately normal ratio. If, however, the plasma as a whole, including the precipitate, is considered, and if the foreign protein is included in the globulin fraction, as seems logical, then the albumin to globulin ratio becomes 21 to 79, which recalls the values in the case of hyperproteinemia associated with multiple myeloma described by Perlzweig *et al.* (7).

DISCUSSION

The possible relation of the peculiar protein found in the blood serum of this patient, as well as in the few cases reported in the literature, to the Bence-Jones protein, found frequently in the urine in multiple myeloma, is a question which demands a consideration of the chemical properties of the latter substance.

The characteristic property, which is useful in the qualitative detection and the isolation of the Bence-Jones protein, is its coagulability at a comparatively low temperature (40 to 60°C.), followed by its resolution at higher temperatures (80 to 100°C.). The erroneous impression is sometimes gathered, however, that the temperatures of precipitation and solution of this protein are physical chemical constants, which can be utilized to effect its clean-cut separation from other proteins. Such is not the case, for it has been shown that the temperatures, both of precipitation and of solution of the coagulum near the boiling point, are influenced by many factors, such as the concentration of the protein itself, of hydrogen ions, of electrolytes, and of other compounds such as urea. If the heating process is repeated, the same phenomena may not be observed, inasmuch as the coagulum, which was formerly completely soluble at the boiling point, may now be only partially soluble. Coagula of Bence-Jones protein, even though formed at appropriate low temperatures, if precipitated in the presence of albumin and globulin, contain admixed with them greater or less amounts of these latter proteins. The precipitability of the Bence-Jones protein by other means, such as the salting out process with various concentrations of different salts, has been studied; but such reactions afford neither a method for sharp differentiation of this protein from other proteins, nor an infallible criterion for judgment of its presence or absence.

In fact, there is abundant evidence that the "Bence-Jones protein," as commonly recognized, is not a single chemical individual, but must be regarded as a *class* of substances which exhibit in common this peculiar coagulation phenomenon. On the other hand, it is unquestionable that a Bence-Jones protein has been isolated from urine, as a chemical individual, in certain cases in which spontaneous crystallization has occurred on standing. Such a case was reported by Bayne-

Jones and Wilson (12). After repeated recrystallization, by means of which the Bence-Jones protein was completely freed from possible traces of serum proteins, they were able to establish, unequivocably, that the Bence-Jones protein was immunologically different from the proteins of normal human serum.

From the above facts the conclusion may be drawn, that, from the point of view of the chemist, it is at the present time impossible to establish the identity and exact quantity of foreign proteins, such as Bence-Jones protein, in serum. Of the cases already mentioned in which Bence-Jones protein was considered to be present in the serum, only in those reported by Ellinger and by Abderhalden was the protein, which coagulated at unusually low temperatures, shown to be, at least partially soluble at high temperatures. In the sera of the cases reported by Jacobson, Short and Crawford, and by Zadek and Lichtenstein, coagulation at low temperatures was observed; but this presumptive evidence for the presence of Bence-Jones protein was not followed up by a study of the behavior of the coagula near the boiling point.

The case here reported, then, is unusual in that hyperproteinemia was associated with the multiple myeloma; and it is unique, inasmuch as the abnormal protein precipitated spontaneously. In fact, this protein must have been in solution in the blood stream in very unstable equilibrium if, indeed, it actually was in solution at all. What relation it bore to the symptoms of disturbed circulation, which were so prominent throughout the patient's illness, must remain a matter for conjecture.

It is of further interest, that the presence of this protein, which differed from the usual type of Bence-Jones protein both in its insolubility and its behavior towards heat, was associated with neither significant kidney damage nor Bence-Jones proteinuria.

SUMMARY

A patient who presented symptoms of coldness, blanching and a peculiar mottling of the extremities, as well as other signs of disturbed circulation, was found to have, in her blood, a voluminous quantity of a substance which invariably was precipitated immediately on withdrawal of the blood from the body. This material was found to be

protein in nature. Autopsy proved that the patient suffered from multiple myeloma.

The chemical nature of this protein is discussed, and its relation to Bence-Jones protein considered. Among approximately 500 case reports of multiple myeloma, abnormality in the blood plasma has been found only in 8 instances. Even in comparison with these cases, however, the case here reported seems to be unique.

BIBLIOGRAPHY

(1) ELLINGER, A.: Das Vorkommen des Bence-Jones'schen Körpers im Harn bei Tumoren des Knochenmarks und seine diagnostische Bedeutung. *Deutsch. Arch. f. klin. Med.*, **62,** 254–278, 1899.

(2) JACOBSON, V. C.: A case of multiple myelomata with chronic nephritis showing Bence-Jones protein in urine and blood serum. *J. Urol.*, **1,** 167–178, 1917.

(3) SHORT, J. J., AND J. R. CRAWFORD: Bence-Jones protein in blood serum leading to detection of multiple myelomatosis. *J. Lab. and Clin. Med.*, **14,** 1092–1093, (August), 1929.

(4) ABDERHALDEN, E.: Ein Fall von Bence-Jones'scher Albuminurie. *Ztschr. f. physiol. Chem.*, **106,** 130–132, 1919.

(5) GABBE: Klinisch-wissenchaftl. Abend im Luitpoldkrankenhaus, Würzburg. Sitzung vom Nov. 1927. *Münch. med. Wchnschr.*, **74,** 2224, 1927.

(6) ZADEK, I., AND LICHTENSTEIN, H.: Zur Klinik und Zytologie der multiplen Myelome. *Folia haemat.*, **45,** 60–74, (August) 1931.

(7) PERLZWEIG, W. A., DELRUE, G., AND C. GESCHICKTER: Hyperproteinemia associated with multiple myelomas. *Jour. Am. Med. Assn.*, **90,** 755–757, (March 10) 1928.

(8) JOHANSEN, A. H.: Hyperproteinemia and multiple tumors, *Hospitalstid.*, **74,** 562, (May 14) 1931.

(9) WALLGREN: *Upsala Lakaref. Forh.*, **25,** 113, 1920.

(10) VENTURI, T.: Di un caso di mielomi multipli. *Riforma med.*, **17,** 675, 1901.

(11) WINTROBE, M. M.: Macroscopic examination of the blood. *Am. J. M. Sc.* (Jan.), 1933.

(12) BAYNE-JONES, S., AND D. W. WILSON: Immunological reactions of Bence-Jones proteins. I. Differences between Bence-Jones proteins and human serum proteins. *Bull. Johns Hopkins Hosp.*, **33,** 37–43, (February) 1922.

BAYNE-JONES S., AND D. W. WILSON: Immunological reactions of Bence-Jones proteins. II. Differences between Bence-Jones proteins from various sources. *Ibid.*, **33,** 117–125, (April) 1922.

STUDIES ON AN UREA-SPLITTING ENZYME FOUND IN THE GASTRIC JUICE[1]

LAY MARTIN

From the Gastro-intestinal and Chemical Divisions of the Department of Medicine of the Johns Hopkins University

The following experiments have been carried out to establish proof of the occurrence of an urea-splitting enzyme in the gastric juice.

METHOD

Gastric juice was obtained from patients who presented themselves for treatment at the Gastro-intestinal Dispensary of the Johns Hopkins Hospital. A duodenal tube was placed in the dependent portion of the stomach of a patient who had fasted for fifteen hours and the position of the tube was verified by fluoroscopy. The entire fasting secretion was removed and placed in the ice-box. A flow of gastric juice was stimulated by a hypodermic injection of histamine (ergamine acid phosphate) in amounts equivalent to .005 milligram per kilo of body-weight. The entire flow of gastric juice was collected by continuous siphonage (or, if necessary, with the aid of syringe) into a narrow container surrounded by ice. After cessation of gastric flow, all the gastric juice was filtered in the ice-box at 7°C. A perfectly clear, limpid fluid was obtained. Very little, if any, change in hydrogen-ion concentration was noted after many days.

Titration of gastric juice was carried out in the usual manner with di-methyl-amino-azobenzene and phenolphthalein as indicators. Hydrogen-ion concentration was determined by the method of Clark and Lubs (1). Cultures were made on dextrose brain agar at pH 6.8. Total nitrogen was determined by the Kjeldahl method (2). Proteins were removed from the gastric juice by the method described in a previous article (3). Non-protein nitrogen was determined on the protein-free filtrate by the method of Folin and Wu (4). The amino acids were also determined on the same protein-free filtrate after adjustment of hydrogen-ion concentration by the method of Folin (5).

[1] Submitted for publication October 13, 1932.

Urease was prepared by the method described by Sumner (6) and kept at ice-box temperature (7°C.). It was freshly made every three or four weeks.

Total ammonia nitrogen. Gastric juice (2.5 c.c.) and soy bean urease (2.0 c.c.) at pH 7.0 were incubated at 45°C. in a water-bath for 15 minutes and the ammonia content determined. This determination represented the ammonia nitrogen of gastric juice plus that obtained from gastric urea by the action of urease and was called total ammonia nitrogen.

Free ammonia nitrogen. The ammonia content was determined upon 2.5 c.c. of gastric juice as free ammonia nitrogen by alkalinization with sodium carbonate. The ammonia was distilled from pyrex digestion tubes through an especially built connection tube and bulb (similar to a connection bulb for a Kjeldahl apparatus) which prevented back flow. The distillate was collected in N/10 hydrochloric acid and the results were determined colorimetrically after nesslerization.

Urea nitrogen. The material from which the ammonia had been distilled was readjusted to pH 7.0. Soy bean urease (2.0 c.c.) was added and the mixture was incubated at 45°C. in a water-bath for 15 minutes. The ammonia was then determined and represented urea nitrogen.

Blanks were run as a control for each method. The error was constant throughout—0.06 mg. nitrogen per 100 c.c. material. In table I a typical protocol for standardization of method is given.

OBSERVATIONS

Effect of unusual temperatures. Gastric juice in sealed glass tubes at pH 2.0, 3.5, 5.0, 7.0 and 8.0 was heated to 60°, 70°, and 100°C. for an hour. The specimens were then incubated at 37.5°C. and estimations of urea and ammonia nitrogen were made at 24, 48, 72, and 96 hours. There was *no change* from the controls.

Gastric juice at pH 2.0, 3.5, 5.0, 7.0, and 8.0 was kept in the ice-box at 7°C. Estimations for urea and ammonia nitrogen *remained as in the controls* when determinations were made at 24, 48, 72, and 96 hours.

Effect of normal body temperature. Observations were carried out on 34 filtered specimens and with but one exception the results obtained

TABLE I

Typical protocol for standardization of method to determine ammonia nitrogen and urea nitrogen

SPECIMEN	TOTAL (UREA AND AMMONIA) NITROGEN	AMMONIA NITROGEN	UREA NITROGEN		TOTAL UREA AND AMMONIA NITROGEN AND UREA, 4.66 MG. PER CENT N
			a. Without added urea	b. With urea, 4.66 mg. per cent N	
	mg. per cent	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*
Synthetic—$(NH_4)_2SO_4$, 2.9 mg. per cent N and $CO(NH_2)_2$, 4.66 mg. per cent N. Average of five tests	7.67	2.98	4.64		
Gastric juice Mixture VIII	7.65	2.99	4.62		
	7.67	2.98	4.63		
Gastric juice Mixture XI	4.98	3.83	1.12		
	4.95	3.87	1.13		
	4.97	3.84		5.75	9.55
	4.94	3.86		5.78	9.61

TABLE II

Changes in urea and ammonia of gastric juice when incubated at 37.5°C. Protocol of Mixture X

TEMPERATURE	N (NITROGEN)	CONTROL	24 HOURS	48 HOURS	72 HOURS
°C.		*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*
37.5	Total ammonia N	7.30	7.32	7.35	7.35
	Urea N	2.07	1.80	1.76	1.70
	Free ammonia N	5.18	5.42	5.50	5.61
7.0	Free ammonia N	5.18	5.16	5.19	5.20
37.5	Non-protein N	25.41			26.95
	Amino acid N	7.50			8.69
37.5	Micro-organisms	0			0
	Yeast colonies	2–3			2–3
	pH	1.9	1.9	2.0	2.0

varied only in degree. The data in table II represent a typical protocol of a mixture of 15 complete extractions of gastric juice, stimulated by histamine, from 15 patients.

When the gastric juice containing free hydrochloric acid was incubated at 37.5°C. at its extracted hydrogen-ion concentration for increasing periods of time, there occurred an *increase in the amount of ammonia and a decrease in the amount of urea.* The increase in one

TABLE III

Change after incubation at 37.5°C. for 48 to 72 hours in amino acids, non-protein nitrogen and proteins of gastric juice

MIXTURE		CONTROL	AFTER INCUBATION	CHANGE	NPN INCREASE MINUS AMINO ACID INCREASE	PROTEINS	
						Control	After incubation
		mg. per cent	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*
IV	NPN	24.18	28.16	3.98		20.84	16.86
	Amino acid	5.60	9.49	3.89	0.09		
VI	NPN	28.02	29.22	1.20		61.98	60.78
	Amino acid	5.69	6.86	1.17	0.03		
VII	NPN	27.45	28.72	1.27		17.77	16.50
	Amino acid	8.83	10.20	1.37	−0.10		
VIII	NPN	30.70	30.90	0.20		25.68	25.66
	Amino acid	7.00	7.00	0.00	0.20		
IX	NPN	19.04	21.30	2.26		22.93	20.67
	Amino acid	4.56	6.78	4.26	0.00		
Remberg	NPN	40.59	41.03	0.42		39.83	39.41
	Amino acid	10.76	11.17	0.41	0.01		
X	NPN	25.41	26.95	1.54		44.39	42.85
	Amino acid	7.20	8.69	1.49	0.05		

approximated the decrease in the other. Furthermore, the total ammonia nitrogen remained relatively stationary throughout the period of observation. There was no change in the control specimens kept in the ice-box.

There was a destruction of gastric-juice proteins during the period of incubation and an increase of non-protein nitrogen. This increased non-protein nitrogen could be accounted for quantitatively by the increased amino-acid nitrogen (see table III).

Effect of micro-organisms. Bacteriologic cultures made from the specimens before and after incubation showed at times an equal number (very few) of yeast colonies and at times a few organisms (colon bacillus, staphylococcus, etc.) were found, but in such small amounts as to be unimportant. The important point is that the reaction was not dependent upon the presence or absence of micro-organisms.

Effect of incubation at 45°C. A small series (5 cases) was carried out by incubating the gastric juice in a water-bath at 45°C. The results varied only in degree as may be seen in a typical protocol in table IV.

TABLE IV

Changes in urea and ammonia of gastric juice at pH 2.0 when incubated at 45°C. Protocol of Mixture VI

TEMPERATURE	N (NITROGEN)	CONTROL	1 HOUR	2 HOURS	3 HOURS	4 HOURS	6 HOURS
°C.		*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*
45.0	Total ammonia N	6.32		6.35		6.35	6.38
	Urea N	2.07		2.00		1.89	1.86
	Free ammonia N	4.22	4.30	4.34	4.46	4.48	4.50
7.0	Free ammonia N	4.22		4.20		4.25	4.25
45.0	Non-protein N	28.02	28.84	28.95		29.00	
	Amino acid N	5.69				6.64	

It is quite obvious that the same type of reaction occurred at both 37.5° and 45°C.; the greater changes are to be seen with the longer duration of incubation. The increase of free ammonia nitrogen in the latter was found to be between 0.3 and 1.5 mg. nitrogen per 100 cc. gastric juice. In the former the greatest increase in six hours did not exceed 0.3 mg. nitrogen per 100 c.c.

Mucus. From the original gastric juice filtered immediately in the ice-box, the mucoid material was collected from the filter paper and sufficient N/50 hydrochloric acid added to bring it into suspension. To 20 c.c. of this mixture were added 5 c.c. of 1 per cent urea solution; after control estimations had been made, it was then incubated at 37.5°C. for 24, 48, and 72 hours. Aliquot portions were analysed for

the various nitrogen constituents. Approximately the same amount of ammonia was produced as by the filtered gastric juice.

Fasting or resting gastric juice. The amount of ammonia produced by incubation of the resting juice which contained free hydrochloric acid was usually found to be slightly greater than that produced in the histamine-stimulated secretions.

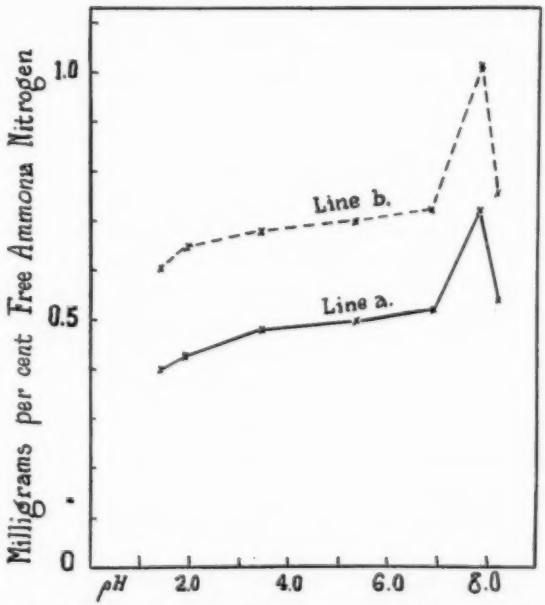

FIG. 1. Line a. Increase of ammonia in normal gastric juice, with associated destruction of urea, after incubation at various hydrogen-ion concentrations. Line b. As in Line a. plus urea, 8.6 mg. per cent nitrogen.

Effect of changes in concentration of hydrogen ion, urea, and reacting agent. By varying the hydrogen-ion concentration of the incubated specimens it was found that the greatest production of ammonia and the greatest destruction of urea occurred at pH 8.0 (figure 1, line a.). At this pH there was no increase in non-protein nitrogen or amino-acid nitrogen. The additional weighed amounts of urea to the gastric juice increased the amount of ammonia produced (figure 1, line b.).

The dilution of the gastric juice with water which contained ammonium sulphate and urea in concentrations equal to that found in the test specimen caused a diminution in the amount of ammonia produced on incubation.

SUMMARY

The observations noted above give fairly conclusive evidence that an urea-splitting enzyme is excreted into normal gastric juice and that its activity is exhibited through a wide range of hydrogen-ion concentration, *i.e.*, from pH 1.4 to at least 8.3. As the greatest action was found to take place at pH 8.0, it would seem that its main seat of activity was within the cells. The investigations of Luck (7) and Rigoni (8) point strongly to its occurrence in the gastric mucosa. The findings in the present paper are additional evidence and in a way corroborate their work. Certainly the gastric mucosa would be the logical location and seat of production for the enzyme.

There seem to be grounds for belief that this urea-splitting enzyme is a material different from that isolated by Sumner (6). The most distinguishing characteristics are its activity over a large range of hydrogen-ion concentration and its much slower reaction.

BIBLIOGRAPHY

(1) Clark, W. M.: The Determination of Hydrogen Ions. Williams and Wilkins, Baltimore, 1928.
(2) Kjeldahl, J.: *Ztschr. Anal. Chem.*, 1883, **22**, 366.
(3) Martin, L.: *Bull. Johns Hopkins Hosp.*, 1931, **49**, 286.
(4) Folin, O., and H. Wu: *J. Biol. Chem.*, 1919, **38**, 81.
(5) Folin, O.: *Ibid.*, 1922, **51**, 377.
(6) Sumner, J. B.: *Ibid.*, 1926, **69**, 435.
(7) Luck, J. M.: *Biochem. Jour.*, 1924, **18**, 814 and 825.
Luck, J. M. and T. N. Seth: *Ibid.*, 1924, **18**, 1227; and 1925, **19**, 357.
(8) Rigoni, M.: *Boll. Soc. Ital. biol. Spe.*, 1929, **4**, 1166; *Arch. Sci. Biol.*, 1930, **15**, 29.